Full-Scale Ozone Ballast Water Treatment for Removal of Marine Invasive Species

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TABLE OF CONTENTS

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1 EXECUTIVE SUMMARY

1.1 Background

The discharge of organisms found in the ballast water of oil tankers and other cargo freighters may be a major threat to public health and the environment around the world. These organisms may cause substantial economic injury in countries in whose water they are discharged. Many of these organisms are not native or established in coastal regions (including ports) where they are discharged with ballast water, and thus are collectively referred to as nonindigenous species (NIS) or invasive species. NIS can substantially disrupt the structure and function of coastal marine ecosystems. The U.S. Coast Guard also estimates that NIS introductions cause approximately $6 billion in economic damage in the United States annually. For example, the U.S. government estimates that over the past 10 years it has cost nearly $4 billion to repair damage caused by the non-indigenous zebra mussel alone, impacting shorelines, water treatment, and power generating stations in and around the Laurentian Great Lakes.

Although many transfer mechanisms (or vectors) have contributed historically to the invasion of coastal habitats by aquatic NIS, shipping has been the vector responsible for most known invasions. The rate of new invasions appears to be increasing over time, and many of these invasions are attributed to the transfer and discharge ships' ballast water. In short, ballast water is contributing strongly to the overall increase in newly detected invasions in coastal marine ecosystems.

Ballast water exchange is currently the only management strategy available for ships to reduce the quantities of non-indigenous coastal organisms in ballast water. Ballast water exchange, or mid-ocean exchange, occurs when ships replace coastal water in their ballast tanks with open ocean water to reduce the abundance of coastal NIS. It is a management strategy that many ships can implement immediately, and which does not require retrofitting or development of new technology.

Ballast water exchange (BWE) has some significant limitations and is viewed generally as a stopgap measure to reduce the risk of invasions. First, it is not always possible to safely conduct an exchange, because of risks to the structure and safety of vessels (especially in heavy seas). Second, even when performed, BWE still leaves a residue of coastal organisms. Third, for many voyages of short duration (e.g., coastwise transits limited to a hours or a few days), sufficient time may not exist to complete ballast water exchange, and the distance from shore may be insufficient to be entirely effective (as described above).

Therefore, efforts are now underway to develop and implement technological alternatives to ballast water exchange. Although many treatment possibilities are being explored, their evaluation is at an early stage and no alternative treatments have yet been approved by state, regional, or federal regulatory authorities. At the present time, the U.S. Coast Guard (as directed by the National Invasive Species Act of 1996) and some states require that alternative treatments be, at a minimum, as effective as BWE. However, no specific guidelines or minimum standards of efficacy currently exist to assess the performance of these alternative treatments.

1.2 Testing the Effectiveness of Ozone as a Potential Treatment Technology

In 1998, British Petroleum Alaska and Nutech O3, Inc. (hereafter referred to as BP and Nutech) undertook the development and testing of ozone gas as a potentially effective alternative method of decontaminating ballast water that contains NIS. A full-scale prototype ozonation system was installed in September 2000 and tested on board the BP-affiliate ship the *S/T Tonsina* (Alaska Tanker Company), a 869-foot, double-hull oil tanker with 12 segregated ballast water tanks, with a total capacity of approximately 11,000,000 gallons (41,365,000 L).

BP and Nutech subsequently partnered with several academic and industrial research institutions to design and implement a rigorous, independent analysis of the ozone system's ability to remove non-indigenous or invasive species from marine ballast water. The study described in this report represents the first of several experimental phases planned to provide a full evaluation of the efficacy of the prototype Nutech ozone system aboard the *S/T Tonsina*. The primary goal of this present (Phase 1) study was to conduct a field-scale test of the operation and efficacy of this ballast water treatment system for removal of a wide range of coastal marine organisms.

The specific objectives of the present study were to:

1) Determine the efficacy of a full-scale ozone system to remove coastal organisms compared to ballast water exchange.

2) Assess the possible environmental risks of discharging ozone-treated ballast water by measuring chemical constituents of the water over time and using whole effluent toxicity testing to assay the latent toxicity of the ballast water at the time of discharge.

3) Obtain operational experience with the prototype ozone system in order to implement further system improvements.

In short, this first phase represents a "proof of concept" for the Nutech ozone treatment system, providing key data needed to address each of the three primary objectives. It is important to recognize the current data, in Phase 1, are limited to a few trials from one port system.

1.3 Experimental Design

This study is the first of several phases, and measured the effects of ozone treatment and ballast water exchange, replicated on multiple dates with ballast water originating from Puget Sound. The experiments were designed to compare changes in treatment tanks over time to those observed in untreated control tanks. Treatment tanks (designated for ozone or ballast water exchange) were filled from the same source as untreated control tanks and all tanks were sampled at fixed time points throughout the same experiment.

Three ozone experiments and two ballast water exchange experiments were conducted. Including a third tank as a control, ballast tanks were filled at the same time and location to obtain a direct comparison between the efficacy of exchange and ozonation. Samples were

collected at multiple time points, including before and after treatment, from each tank using several access locations (manways or Butterworth® openings) on the deck of the ship. Treatments were as follows: No. 3 wing port (ozone treatment); No. 3 wing starboard (air-sparged control); and No. 4 port (ballast water exchange). Samples were used to measure changes in biota and water chemistry over time, as described below.

Effects of treatment on biota were measured in two ways. First, for organisms entrained in the ballast tanks, samples were collected from treatment and control tanks at least before and after treatment, and sometimes at intermediate time points, to compare changes in concentration and condition of resident organisms between treatments. This approach was used to measure effects of ozone and ballast water exchange treatments on bacteria, phytoplankton, and zooplankton. Second, for larger organisms (which are rare and more difficult to sample), a defined number of individual organisms were placed in various types of cages to measure the effect of ozone treatment. This second approach was used for fish, crabs, mysids, and amphipods. These caged organisms were placed in ozone treated and control tanks to compare mortality rates over time; a similar approach was not used in the BWE tanks, due both to the turbulence associated with this treatment and the mode of action, which was considered to be primarily achieved through removal and not mortality.

One preliminary and three full experiments were conducted over the course of one year. The preliminary test, designed to provide data for the full scale testing, provided information on the chemical reactions of ozone, including by-product formation and their effects on bacteria. Experiment 1 closely mimicked the ozone dosage that could be achieved on the *S/T Tonsina* during routine operations. During a typical 3.5-day voyage, the ozone system would apply 0.62 mg/L/hours ozone to the 2,850,000 L of each segregated ballast water tank in the vessel for a duration of five hours. This would be achieved by treating the 12 segregated ballast water tanks separately. During experiment 1, the ozone-loading rate was 0.59 mg/L/hours and lasted 5 hours. Experiment 2 achieved an ozone-loading rate of 0.86 mg/L/hours that resulted from improved operation of the ozone generator. In experiment 3, where only the vertical portions of the tanks were treated and the experiment lasted for 10 hours, an ozone-loading rate of 1.35 mg/L/hours was achieved. In Experiments 2 and 3, much larger amounts of ozone were purposely directed to the tank compartments that were sampled.

1.4 Results

1.4.1 Efficacy of Ballast Water Exchange

Ballast water exchange removed an average of 64% of the target animals measured in the first two exchange experiments (Figure 1.1). For each experiment, 5 coastal organisms were selected, on the basis of their abundance and restricted coastal distribution, to provide a quantitative measure of exchange efficacy. Figure 1.1 indicates the percent reduction observed in the ballast water exchange treatment relative to the control treatment of each of the target taxa. The data are displayed by experiment, indicating the variation observed among taxa. Despite considerable variation among taxa, the mean efficacy among taxa was similar between experiments: 59 % and 69 %.

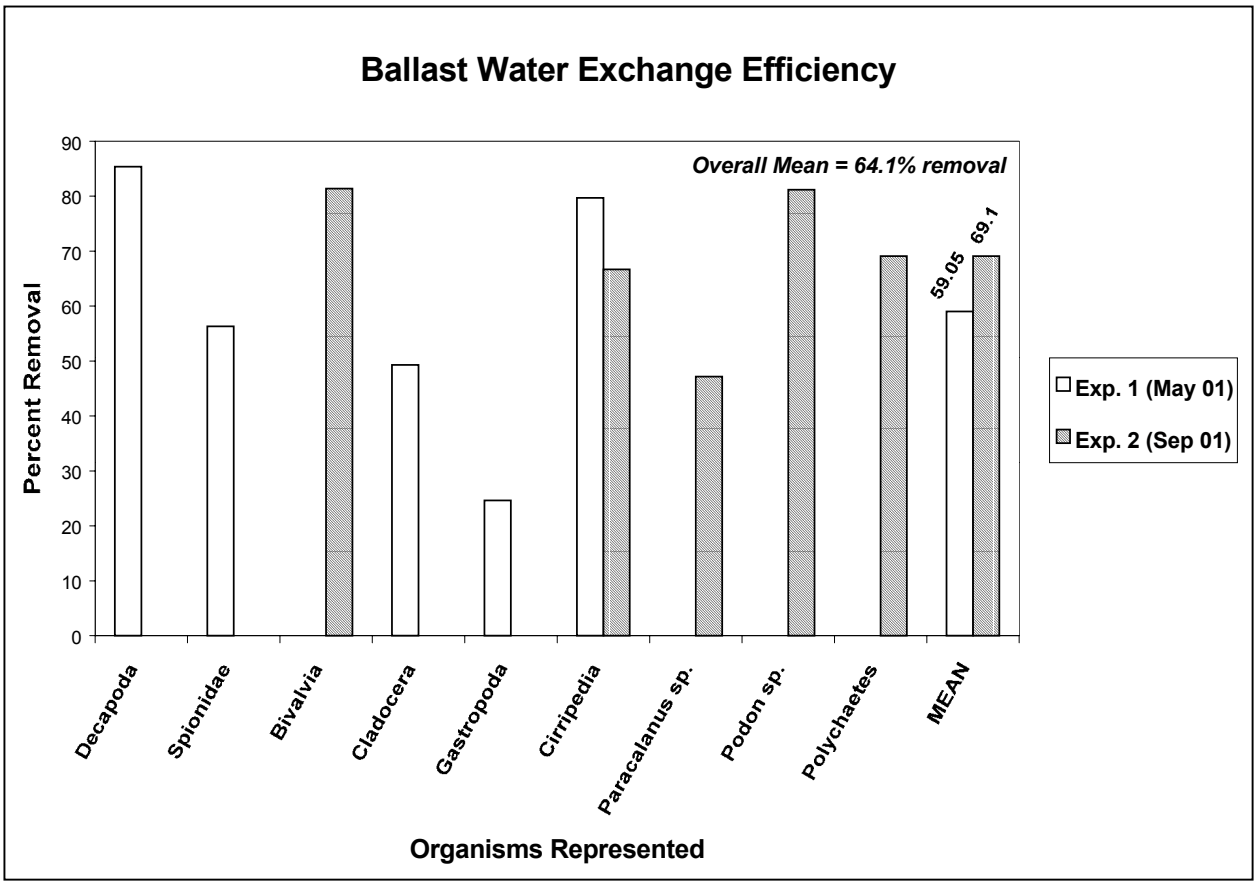


Figure 1.1. Summary of percent removal of marine organisms using ballast water exchange.

The efficacy of BWE, measured aboard the *S/T Tonsina*, was considerably lower than some proposed regulatory targets of 95 %. This level of reduction also appeared lower than that measured on other vessels, resulting perhaps from the structural complexity of the *S/T Tonsina's* ballast tanks relative to the other vessels examined to date.

The direct comparison of BWE and ozone treatment on the same vessel is critical in evaluating the ozone treatment effectiveness. Moreover, our results (1) underscore the variation the can exist within ship type, and (2) suggest the level of "kill" needed for ozone treatment to surpass ballast water exchange aboard the *S/T Tonsina* may be lower than that for other vessels.

1.4.2 Ozone Chemistry

In seawater where there is a significant concentration of bromide ion (Br^-), ozone is catalytically destroyed with a half-life of five seconds. As expected, there was no ozone observed in any of the ballast water samples we analyzed. Therefore, ozone *per se* can be considered a good oxidant for the disinfection of marine ballast water because it is not chemically persistent.

Bromate ion (BrO_3^-) was never detected at measurable levels in the treated ballast water, suggesting that the lower pH of the coastal water favored the formation of hypobromous acid (HOBr). Ozone and its residuals apparently did react with naturally occurring organic matter resulting in the formation of modest concentrations of bromoform in our experiments. The appearance of bromoform, and the fact that no bromate ions (or chloroform) were detected in any of the experiments, indicates that bromine (represented by hypobromous acid/hypobromite ions, or $HOBr/OBr^-$) was formed in significant quantities during the ozonation process.

Concentrations of ozone-produced oxidants (i.e., bromine) were measured in ballast water using an electrode measurement of Oxidation-Reduction Potential (ORP), and a chemical measurement for Total Residual Oxidants (TRO). Ozonation increased ORP levels up to a plateau of ca. 700-800 millivolts (mV), which is consistent with seawater disinfection targets used by commercial marine exhibit aquaria. TRO levels exceeded limits of analytical detection (4 mg/L as chlorine equivalents) in most of the experiments on board the *S/T Tonsina*. The scientific literature suggests that even 4 mg/L TRO should exceed concentrations known to be acutely toxic (e.g., 1-2 mg/L) to many marine organisms.

1.4.3 Efficacy of Ozone Treatment in Ballast Water Tanks

Figures 1.2 and 1.3 summarize the efficacy of ozone treatment for different organisms, for the different experiments, and time of ozonation at the time of sampling. Figure 1.2 summarizes the results of "killed" organisms while Figure 1.3 summarizes the total for the killed and moribund organisms. Efficacy for each organism is estimated as (a) the percent reduction in initial concentration for bacteria, microflagellates and dinoflagellates or (b) the percentage of sampled organisms that were dead or moribund for zooplankton, sheepshead minnow and mysid shrimp. The results are compared to the 64 % BWE efficacy (i.e., percent removal) as measured for zooplankton on the *S/T Tonsina* (Section 1.4.1). The percent removal for each group is shown, along with an indication (denoted by bars labeled with *) of whether percent removal of that particular organism by ozone was greater than that of mean BWE performance on this vessel.

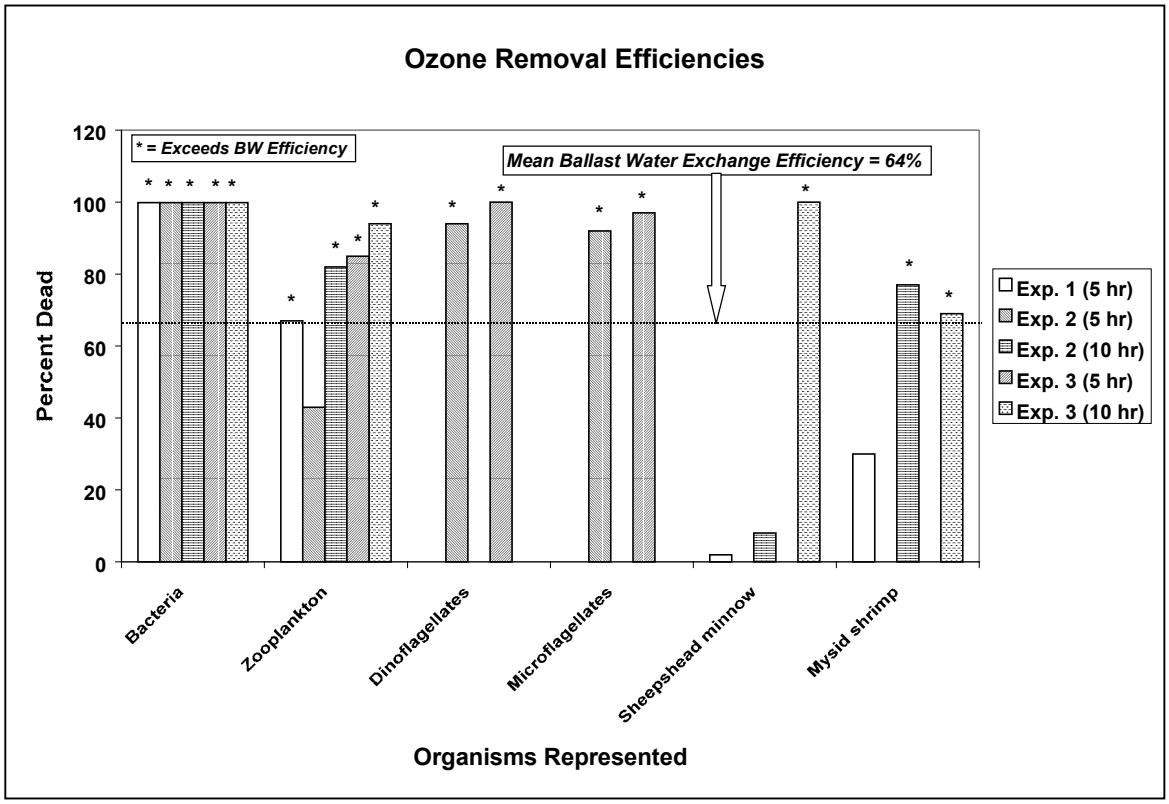


Figure 1.2. Percent mortality in ozone treatments vs. 64% mean organism removal efficiency from ballast water exchange on the *S/T Tonsina.*

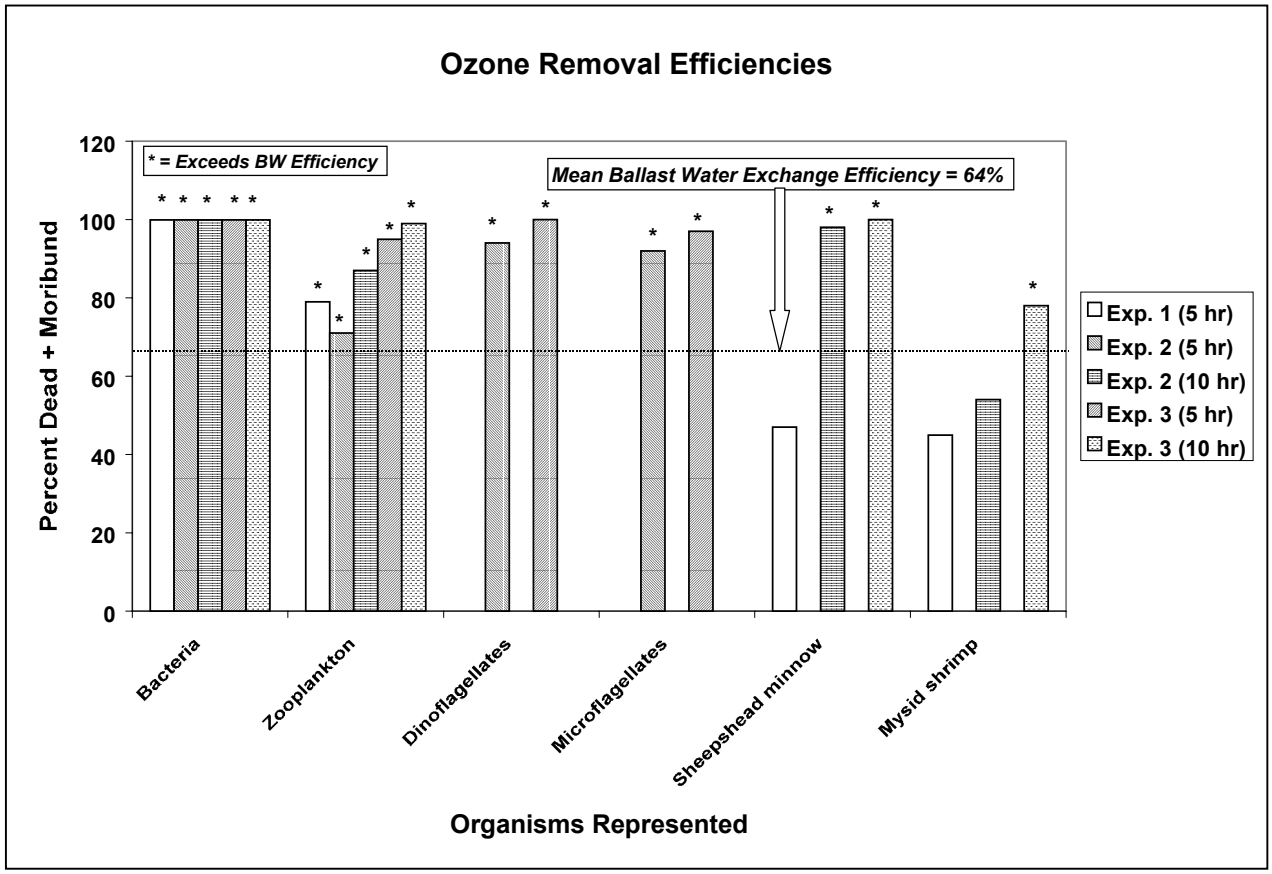


Figure 1.3. Percent dead + moribund in ozone treatments vs. 64% mean organism removal efficiency from ballast water exchange on the *S/T Tonsina*.

For the ozone treatment, the following results were observed (relative to the paired control treatment):

1. The concentration of culturable bacteria declined 99.9 %.

2. The zooplankton examined were determined to be 71-99 % dead or near death (moribund).

3. The concentration of vegetative cells for dinoflagellates and microflagellates declined 92 – 100 %. The effects of ozonation on diatoms have not yet been measured.

4. Results for larger, caged organisms were more variable. Among experiments, mortality was as follows: 2-100 % for sheepshead minnows, and 30-77% for mysid shrimp (Figures 1.2). For the sheepshead minnow and mysid shrimp, many organisms appeared moribund and may have been seriously impaired by the treatment, potentially increasing the overall effect of the ozone treatment (Figure 1.3).

5. Mortality rates for benthic organisms (e.g., amphipods and shore crabs) tended to be low. However, in contrast to the sheephead minnows and mysid shrimp, the amphipods and crabs did not exhibit noticeable signs of stress that could result in long-term mortality.

6. The efficacy of ozone treatment generally surpassed that for BWE for bacteria, zooplankton, and phytoplankton.

7. For the larger organisms, it is presently not possible to compare the results of ozone treatment to BWE. We presume exchange would reduce the concentration of these organisms, but it remains difficult to obtain such data for large, mobile organisms.

8. Studies using known numbers of caged organisms suspended in ballast water tanks generally confirmed the level of ozone efficacy, as well as the relative sensitivity of various marine species.

1.4.4 Laboratory Toxicity Tests

The effect of various ozone exposure concentrations and durations on marine organisms was also studied using controlled laboratory experiments. Median lethal concentrations (i.e., [1]LC50) for all but one species exposed to ozonated artificial seawater in the laboratory ranged from 698 - 768 mV ORP, and from 1.29 - 2.93 mg/L TRO. 50% mortality was never achieved for the amphipod, *Leptocheirus plumulosus*. These data were consistent with results from the caged organism studies in which mortality (at least for mysid shrimp) also was strongly correlated to ORP measurements. Therefore, ORP measurements ranging from 700-800 mV

[1] LC50 represents the concentration of a chemical that causes 50% mortality in an acute toxicity test

appear to be associated with significant acute mortality in a variety of marine species both in the field and in the laboratory.

Furthermore, the relative sensitivity of test species exposed to ozone (as measured by ORP) was similar in both the field and lab experiments. In the caged studies, the sheepshead minnow *C. variegatus* was the most sensitive species, followed by mysids (*A. bahia*) and amphipods (*R. abronius*). In the laboratory, LC50 values for *C. variegatus* were indeed lower than *A. bahia*, suggesting that the sheepshead minnow was slightly more sensitive with respect to ORP exposure. One of the amphipod species tested in the laboratory (*L. plumulosus*) was less sensitive to ORP than either sheepshead or mysids. Thus, laboratory studies provided a realistic indication of ozone toxicity to various species.

1.4.4.1 Toxicity of Ballast Water Following Ozonation

A major concern following treatment of ballast water with any biocide is the discharge of potentially toxic chemicals to the environment. For ozonated seawater, bromine is the residual oxidant most likely to exist for any extended period of time, in concentrations potentially harmful to marine organisms. Therefore, we conducted a series of laboratory tests with ozonated seawater generated either from the main *S/T Tonsina* experiments, or using a similar laboratory-scale ozone generator. The goals of these studies were to evaluate whether ozone residuals may be toxic in seawater and whether this toxicity may persist over time. The following discussion is a summary of those tests.

1.4.4.2 Whole Effluent Toxicity Tests

As part of the regulatory process for the approval of a ballast water chemical treatment process, the treated water will likely need to be screened for potential toxicity using standard whole effluent toxicity (WET) tests. WET tests are widely conducted as part of routine monitoring of wastewater discharges regulated under the federal Clean Water Act. Results of the WET tests using ozone-treated ballast water with the mysid shrimp, *Americamysis bahia,* and the topsmelt, *Atherinops affinis*, indicated that ozonation byproducts were stable enough to cause toxicity in ballast waters 1-2 days after ozonation and at dilutions of from 30 – 80 %. However, no chemical measurements were conducted in these tests to quantify concentrations of ozone-produced oxidants.

1.4.4.3 Latent Toxicity Tests

To validate the WET test results, mysid shrimp were exposed to ozone (using 4-5 hours of ozonation) in the laboratory using experiments of similar design to the WET tests. We initiated tests with ozonated waters that were stored for 0, 24 or 48 hours and measured toxicity along with ORP and total residual oxidant (TRO) over time. As expected from the WET tests, residual oxidants did not disappear from ozonated waters held in the dark 24 or 48 hours in a sealed container at 12 ºC. All organisms died when exposed to 50, 75, (diluted) or 100 % (non-diluted) water that had been ozonated and stored either 0, 24 or 48 hours. In treatments where 100 % mortality occurred by 24 hours, the ORP was greater than 720 mV, and TRO greater than 1.76 mg/L.

We also evaluated whether relatively short-term ozonation might generate sufficient oxidant (i.e., bromine) to cause acute mortality to mysid shrimp transferred to clean seawater 1-2 days following ozonation. Limited mysid mortality (30-60%) occurred within the 1.5 hours of ozone exposure in laboratory experiments where TRO concentrations exceeded 4.0 mg/L. However, 100% mortality was observed in those survivors 48 hours after transfer to clean seawater. No mortality was observed within 1.5 hours of ozonation, or at 24 hours post-exposure when TRO measurements were less than 1.0 mg/L, but 60% mortality occurred in these same treatments after 48 hours of post-exposure. Therefore, it appears that sufficient amounts of bromine oxidants built up in the ozonated water over 1.5 hours to have induced both immediate and, to an even greater extent, delayed mortality after transferring organisms to clean water (up to 48 hours later).

The presence of bromine thus may cause both immediate and delayed toxicity to marine organisms even after relatively short periods of ozonation. Preliminary experiments suggested, however, that this residual bromine may be easily removed using commonly available reducing agents such as sodium thiosulfate, and thus this could remove toxicity from ozonated ballast waters prior to discharge. Bromine also is likely to be quickly destroyed (i.e., chemically reduced) upon discharge into marine surface waters, and so may be of only limited environmental/regulatory concern for ballast water discharge. Additional study is warranted to verify this conclusion.

1.5 General Conclusions and Recommendations

Results from this (Phase I) study, using the prototype system on board the *S/T Tonsina,* suggest that ozonation can be effective at removal of many coastal organisms from full-scale ballast tanks and may compare favorably with BWE. Key conclusions of our study include:

1. Using this prototype system, 5-10 hours of ballast water ozonation resulted in a 71-99% reduction of selected marine phytoplankton, zooplankton and bacteria. The results depended upon the individual organism and the amount of ozone gas delivered to individual ballast water tanks over time.

2. Large, mobile organisms (especially benthic crabs and amphipods) appeared to be relatively resistant to ozone treatment compared to planktonic organisms.

3. Our experiments may have underestimated the efficacy of ozone treatment resulting from the possible residual toxicity of bromine over time. Some organisms appeared affected by the initial treatment and may succumb over time, however, such effects are not included in our analysis. Additional study under field conditions is warranted to test for such effects.

4. The efficacy of ozone treatment to reduce planktonic organisms was as good as that of BWE aboard the same vessel for which empty-refill exchange resulted in an average reduction of 64% for zooplankton.

5. Both field and laboratory experiments suggested that significant organism mortality can be achieved once concentrations of ozone-produced oxidants reach 1 – 3 mg/L (as chlorine equivalents), or when oxidation-reduction potential reaches levels of 700 – 800 mV. Once further validated, such toxicity thresholds could be used to help develop control targets for aiding the routine operation of ozone systems.

6. Our preliminary results suggested that bromine was the ozone-produced oxidant that was responsible for organism mortality. Furthermore, bromine may persist at toxic concentrations in ballast waters 1 - 2 days following ozonation depending on storage conditions and exposure to sunlight.

2 INTRODUCTION

2.1 The Problem

The worldwide transfer and introduction of nonindigenous species (NIS), or invasive species, by human activities is having significant and unwanted ecological, economic and human-health impacts (e.g., OTA 1993, Wilcove et al. 1997, Pimentel et al. 2000). Although most attention to date has focused on invasions in terrestrial and freshwater habitats, it is evident that NIS invasions have become a potent force of change in coastal marine ecosystems. Roughly 400 marine and estuarine NIS are known to have been established in North America alone and over 200 of these species can occur in a single estuary (Cohen and Carlton 1995, Ruiz et al. 1997, 2000a). Some of these species have become numerically or functionally dominant in invaded communities, where they have significant impacts on population, community and ecosystem-level processes (e.g., Cloern 1996, Crooks 1999, Ruiz et al. 1999, Grosholz et al. 2000).

Although many transfer mechanisms (or vectors) have contributed historically to the invasion of coastal habitats by NIS, shipping has been the vector responsible for many of the known invasions (Carlton 1979, Carlton and Geller 1993, Cohen and Carlton 1996, Hewitt et al. 1999, Ruiz et al. 2000a). Furthermore, the global movement of ballast water now appears to be the single largest transfer mechanism for marine NIS. Since the 19th century, ships have used ballast water for stability, discharging water both at ports of call and en route (Carlton 1985). Ports receive relatively large volumes of ballast water originating from source regions throughout the world. For example, the United States and Australia each receive annually over 79 million metric tons of ballast water on ships arriving from foreign ports (Kerr 1994, Carlton et al. 1995). A taxonomically diverse community of organisms is entrained and transported within ballast tanks (e.g., Carlton and Geller 1993, Smith et al. 1999, Hines and Ruiz 2000, Ruiz et al. 2000b), resulting in many successful invasions of nonindigenous species at ports throughout the world.

BWE or mid-ocean exchange is currently the only management strategy available for ships to reduce the quantities of non-indigenous coastal plankton in ballast water (National Research Council 1996). Ships practice two types of BWE that replace coastal water with oceanic water, reducing the initial concentration of coastal organisms (i.e., those that are most likely to invade a port). Flow-Through Exchange occurs when water from the open ocean is pumped continuously through a ballast tank to flush out coastal water, and Empty-Refill Exchange occurs when a tank is first emptied of coastal water and then refilled with oceanic water.

The National Invasive Species Act of 1996 (NISA) created a program in which vessels arriving from outside of the Exclusive Economic Zone (EEZ) voluntarily conduct open-ocean exchange, or use an approved alternate treatment of ballast water permitting ballast tanks to be discharged in U.S. ports. More recently, individual states (e.g., California, Maryland, Oregon, Washington and Virginia) have passed and implemented similar laws, sometimes making this management mandatory.

BWE is viewed generally as a “stop-gap” measure to reduce the risk of invasions. It is a management strategy that many ships operators can implement immediately and does not require retrofitting or development of new technology. However, ballast exchange has some significant

limitations. First, it is not always possible to safely conduct an exchange in high seas. Second, some risks to the structure and safety of vessels in "bad weather" exist and may prevent exchange. Third, the data for the efficacy of BWE are incomplete; however, in any case ballast exchange leaves a residue of coastal organisms in the ballast tank where they contaminate the exchanged water.

Efforts are now underway to develop and implement technological alternatives to BWE. Although many treatment possibilities are being explored (e.g., NRC 1996, Hallegraeff 1998, http:/www.invasions.si.edu), their evaluation is at a very early stage and no alternative treatments have been approved.

At the present time, the U.S. Coast Guard (as directed by NISA) requires that alternative treatments be at least as effective as BWE. However, there exist no specific guidelines to assess the performance of treatments. In Appendix B, we present a conceptual framework for evaluation of alternative treatments and, based on this framework, we designed and executed a study protocol to measure the efficacy of ozonation as a specific treatment system. This report presents the results of pilot studies designed to evaluate the efficacy of ozonation as an alternative system for removal of nonindigenous species from marine ballast water.

2.2 Goals and Objectives

The goal of the study conducted in autumn 2001 was to conduct a field-scale test of the operation and effectiveness of the Nutech ozone ballast water treatment system. While preliminary studies (Section 2) suggested that the process was likely to be effective, its performance with respect to higher organisms at the field scale was as yet untested. Therefore, the present study evaluated the efficacy of ballast water ozone treatment when applied to a wider range of aquatic organisms in a full-scale oil tanker installation, using ballast water collected in the Puget Sound region prior to the *S/T Tonsina's* return to Valdez, AK. Three tests were conducted: one involving 5 hours of ozonation on September 24 and two involving 10 hours of ozonation on November 2 and November 4, 2001.

The specific objectives of the study were:

- To evaluate the chemical and biological quality of ballast water in the treated *vs*. control ballast water tanks over the course of the ozonation periods. Several types of data were collected to assist in this evaluation:
    - Concentrations of ozone and its residuals, along with basic water quality characteristics;
    - The abundance and diversity of several taxa of marine biota normally entrained into ballast water tanks (e.g., bacteria, zooplankton, phytoplankton); and
    - The survival of caged marine organisms of known identity and abundance
- To estimate the reduction of selected organisms by ozone treatment as compared to similar measures for BWE.
- To evaluate the potential toxicity (via ozone and/or its by-products) of post-treatment ballast water prior to discharge using whole effluent toxicity (WET) tests.

The current study (Phase I) was intended as a proof-of-concept for ozone treatment that will be further tested in additional phases. Using grant money from the U.S. Fish and Wildlife Service and the NOAA National Sea Grant College Program, the next phases will evaluate more complex aspects of spatial complexity in ozonation effectiveness and other possible sources of variation including water quality and composition of the entrained biotic community (Section 9).

3 LITERATURE REVIEW

3.1 Efficacy of Ballast Water Exchange

The exchange of ballast water taken on board while in port and the replaced water with oceanic ballast water during a voyage is the only available method for ship owners to reduce the transfer of aquatic organisms. BWE is also the only method specifically supported by national and international regulations at this time. However, the efficacy of BWE is limited because it is not possible to completely replace all of the water, sediments and associated biota resident in ballast tanks during an exchange. Also, the stress on hulls created by BWE can make it unsafe for some ships to undertake exchanges, especially in heavy seas.

The current standard for BWE procedures promulgated by the International Maritime Organization (IMO) is 300 % exchange by volume for the flow-through method and 100 % exchange for the empty-refill method (IMO Resolution A.868(20), 1997). These standards provide a theoretical level of at least 90 % replacement of coastal water by oceanic water, but this has not been adequately validated by field studies (Hines et al, 2000). The exchange of ballast water does not necessarily imply that an equivalent exchange of organisms occurs. In fact, the efficacy of organism removed will vary considerably among different organisms depending upon their size, mobility, behavior and whether the organism is associated with the water column or the benthos (the sediment that may accumulate in the bottom of the ballast tanks).

Because of the limitations of BWE, alternative methods for treating ballast water are actively being developed and tested. In the United States, the U. S. Coast Guard encourages such development and will approve treatment methods demonstrated to be at least as effective as BWE (USCG, Federal Register: Page 17782-17792, April 10, 1998). However, the effectiveness of BWE is poorly documented and not well understood (e.g. Everett, 2001). This is due not only to the relatively few studies that have attempted to document the effectiveness of BWE, but also to the varying methodologies which have been used in those studies.

Ten such studies undertaken by various authors between 1988 and 2000 and compiled by the U.S. Coast Guard attempted to document the effectiveness of more than 100 BWEs conducted by bulk carrier and container ships (Everett, 2000, unpublished report). These studies measured ballast exchange effectiveness in terms of volumetric water replacement at between 87.8% and 99%, and/or in terms of removal of selected planktonic organisms at between 48% and 100%, with occasionally significant variation between the two types of measurement.

Another study was undertaken in 1998 and 1999 on oil tankers similar to the *S/T Tonsina*, which were engaged in trade patterns nearly identical to the *S/T Tonsina's* (transport of Alaska North Slope crude oil from Valdez, Alaska to refineries on the U.S. West Coast). In six

exchange experiments aboard these tankers, volumetric exchange of ballast water was measured, using rhodamine dye as a tracer, to compare 300% flow-through exchange in one tank to 100% empty-refill exchange in another. In these comparative experiments, both types of exchange appeared to achieve very high volumetric water replacement percentages (~99%), although the empty-refill method was able to accomplish this after one volume was exchanged rather than the two or three required by the flow-through method (Ruiz et al., unpublished data).

Although these studies are helpful in illustrating the range of effective BWEs, any generalization of these effects across ships and organisms is premature. This results both from the limited number of measurements and the diversity in methodology that limits direct comparison.

Thus, to effectively compare the efficacy of any treatment (e.g. ozone) to BWE it is necessary to measure concurrently the performance on the same vessel with the same methods. We included BWE experiments in the study design for this project using water that was taken up at the same time and place as water used in the ozone experiments so that direct comparability between the two treatment methods could be achieved.

3.2 Ozone Chemistry: A Brief Review of Fresh and Marine Waters

Ozone has been used as a disinfectant since the late 1800's. It is used widely in Europe in drinking water treatment and to a lesser extent in the U.S. (Hoigne, 1998). It is an oxidant and biocide and is unstable in water (Langlais et al., 1991).

An excellent discussion of ozone decomposition in water that does not contain bromide has appeared in a publication authored by Staehelin and Hoigne, 1985. Fundamentally, ozone decomposition is a base-promoted decomposition with a half-life of 20 seconds at pH = 9. With decreasing pH, the half-life increases by an order of magnitude for each decrease in one pH unit. Ozone decomposition is a chain reaction that involves the formation of the hydroxyl radical, ·OH. The initial reaction of ozone with OH^- results in the formation of superoxide anion radical $O_2^{-}\cdot$. The $O_2^{-}\cdot$ is in equilibrium with its protonated form $HO_2\cdot$ with a pK_a (equilibrium constant) = 4.8 (Bielski et al., 1985). It was noted that the presence of organic and some inorganic compounds promoted the decomposition of ozone. These reaction by-products are transient species and may be involved in the disinfection process, but would not persist in solution.

The biggest difference between ozone chemistry in water treatment and treating marine ballast water is the presence of bromide ion in seawater (Oemcke and van Leeuwen, 1998). Bromide ion catalytically decomposes ozone according to Figure 3.2.3 (von Gunten and Oliveras, 1998) and other studies (Salhi and von Gunten, 1999; von Gunten and Hoigne, 1992; von Gunten et al., 1996; von Gunten and Oliveras, 1997; Pinkernell et al., 2000; Pinkernell and von Gunten, 2001; von Gunten et al., 2001). Two relatively stable by-products are formed when ozone is used to treat seawater, bromate ion and bromoform. The formation of these by-products is through the oxidized bromide ion (bromine). In seawater, bromine rapidly forms hypobromous acid, which is in equilibrium with hypobromite ion. It is also possible to form monobromamine if the concentration of ammonia is sufficiently high. Monobromamine is unstable and will decompose to ammonia and bromide ion (Hofman and Andrews, 2001).

The following equations describe the chemistry shown in Figure 3.2.3. Where they are known, reaction rate constants or equilibrium constants are included (Haag and Hoigne, 1983; von Gunten and Hoigne, 1994).

$O_3 + Br^- \rightarrow O_2 + OBr^-$ $k = 160 \pm 20\ M^{-1}\ s^{-1}$ (3.2.1)

$O_3 + OBr^- \rightarrow 2O_2 + Br^-$ $k = 330 \pm 60\ M^{-1}\ s^{-1}$ (3.2.2)

$OBr^- + H_3O^+ \rightarrow HOBr + H_2O$ $k = 2.06 \times 10^9\ M^{-1}\ s^{-1}$ (3.2.3)

$HOBr + H_2O \rightarrow OBr^- + H_30^+$ $pK_a = 8.8 - 9.0$ (3.2.4)

$2O_3 + OBr^- \rightarrow 2O_2 + BrO_3^-$ $k = 100 \pm 20\ M^{-1}\ s^{-1}$ (3.2.5)

$HOBr + NH_3 \rightarrow NH_2Br + H_2O$ $k = 7.5 \times 10^7\ M^{-1}\ s^{-1}$ (3.2.6)

Haag and Hoigne (1983) and von Gunten and Pinkernell (2000) suggest that no reaction of HOBr with ozone occurs. The pK_a of 8.8 (the pH at which there exist equal amounts of HOBr and OBr^-) suggests that at normal seawater pH, a significant proportion of HOBr would be observed. Therefore, it is possible that "bromine" can accumulate in ozonated ballast water.

Crecelius (1979) studied the ozonation of seawater and measured the formation of bromate ion and total residual oxidant, TRO, (Figure 3.2.4). The concentration of both reaction products increased with the time of ozonation. The TRO showed an initial increase and subsequent decrease in concentration presumably due to back reactions as the reaction time increased. From the chemical cycle shown in Figure 3.2.3 it is likely that the measurement of TRO is entirely made up of $HOBr/OBr^-$. No oxidized forms of chlorine are possible under ozonation treatment conditions.

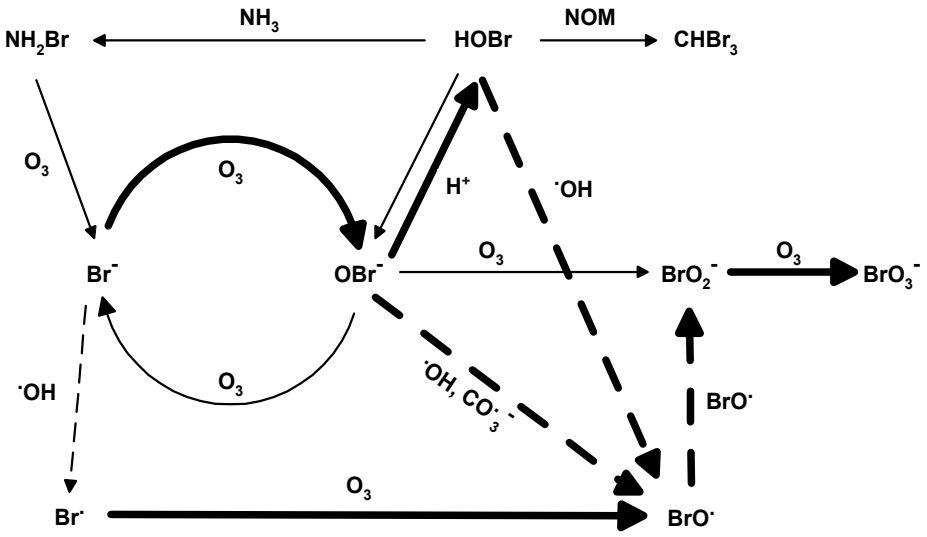


Figure 3.2.3. Reaction pathways for the decomposition of ozone in seawater with the formation of reaction by-products bromate ion and bromoform shown (Driedger et al., 2001 (Reprinted with permission from Elsevier Science); Haag and Hoigne, 1983; von Gunten and Hoigné, 1994; von Gunten and Oliveras, 1998).

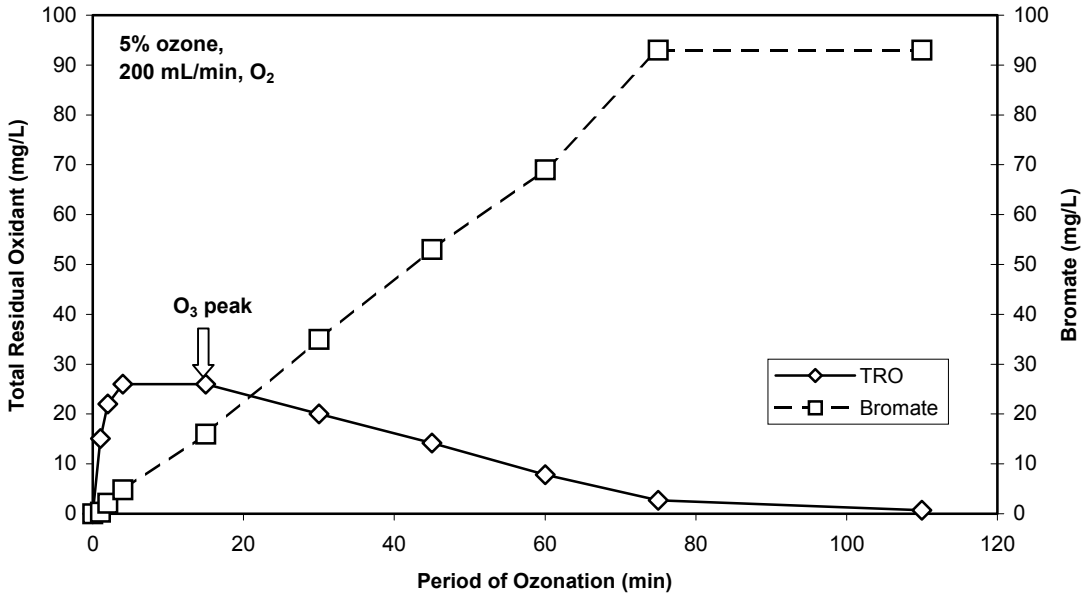


Figure 3.2.4. The ozonation of seawater with increasing time showing the formation of the reaction by-products, bromate ion and total residual oxidant (from Crecelius, 1979).

Therefore, any study designed to evaluate ozone as a possible treatment for ballast water using oceanic sources of water must include the analysis of the two oxidants (Table 3.2.1) and the two reaction by-products (Table 3.2.2).

Table 3.2.1. Oxidants measured when evaluating ozone for ballast water treatment.

Indicator	Definition	Potential significance for ballast water monitoring.
Ozone	O_3	Primary oxidant for ballast water treatment.
Bromine	$HOBr/OBr^-$	Results from the oxidation of bromide ion.

Table 3.2.2. Reaction by-products analyzed when evaluating ozone for ballast water treatment.

Indicator	Definition	Potential significance for ballast water monitoring.
Bromate ion	BrO_3^-	Results from the ozonation of bromide ion in salt water.
Bromoform	$CHBr_3$	Results from the reaction of bromine with naturally occurring organic mater in water used for ballast.

Oxidation-reduction reactions occur during the disinfection process. Thus, the ORP of ozonated water can provide an overall estimate of the oxidizing potential of the water. ORP has been used successfully in controlling ozone levels in aquaria (Aiken, 1995). This measurement may afford a control option for the ozone process. Aiken (1995) reports that for typical use of ozone in aquaria an ORP reading of 400 mV in seawater relates to an ozone dose of 0.02 mg/L, and a reading of 800 – 1000 mV inside the ozone contact chamber (of aquaria) would result in a water that was disinfected.

3.3 Toxicity of Ozone and Its By-products in Seawater

Ozone toxicity tests have been conducted for several marine taxa, including microalgae, invertebrates and vertebrates (Table 3.3.1). Unfortunately, the wide range of exposure conditions and test endpoints used among all of the marine toxicity tests makes it difficult to quantify a general effect concentration for ozone. Furthermore, analytical measurements taken in most tests were not specific to ozone, but rather are expressed as TRO, or "ozone-produced oxidants." "Ozone" toxicity is thus most correctly expressed as a function of TRO, rather than O_3 *per se.*

Many of the toxicity tests exposed organisms to ozone gas diffused in water for relatively short periods of time (e.g., 5-15 minutes), then measured acute toxicity over typical time periods (e.g., 24-96 hours). In these tests, substantial mortality (i.e., 50-100 % mortality) was observed for microalgae, crabs and lobster at concentrations ranging from 0.14 – 1.0 mg/L of TRO (Table 3.3.1). In most of these tests, TRO was measured using a standard amperometric titration reported as chlorine equivalents (Moffett and Shleser 1975, Toner and Brooks 1975). Crab zoea (free swimming planktonic crab larvae) and megalops (crab larval life stage after zoea stage) qualitatively were more sensitive to TRO than the microalgae or lobster, but no quantitative toxicity endpoints were derived in these tests.

Ozone toxicity tests with striped bass and white perch were conducted using flow-through test systems to deliver more reliable and consistent ozone exposures (Table 3.3.1; Block et al. 1978, Hall et al. 1981, Richardson et al. 1983). For striped bass, LC50s (i.e., concentration that kills 50 % of the organisms) ranged from 0.06 – 0.2 mg TRO/L depending on the life stage tested and length of exposure (Hall et al. 1981). Eggs were the most sensitive life stage when reared in freshwater (LC50 = 0.06 mg TRO/L), but fingerlings were most sensitive in seawater if the test was run for 96 hours (LC50 = 0.08 mg TRO/L). Slightly higher concentrations (0.15 – 0.4 mg TRO/L) induced 100% mortality (i.e., LC100) to striped bass fingerlings. In contrast to striped bass, TRO was slightly less toxic to white perch with LC50 values ranging from 0.2 – 0.38 mg TRO/L (Richardson et al. 1983), and an LC100 of 0.8 mg TRO/L after a 6-hour exposure (Block et al. 1978).

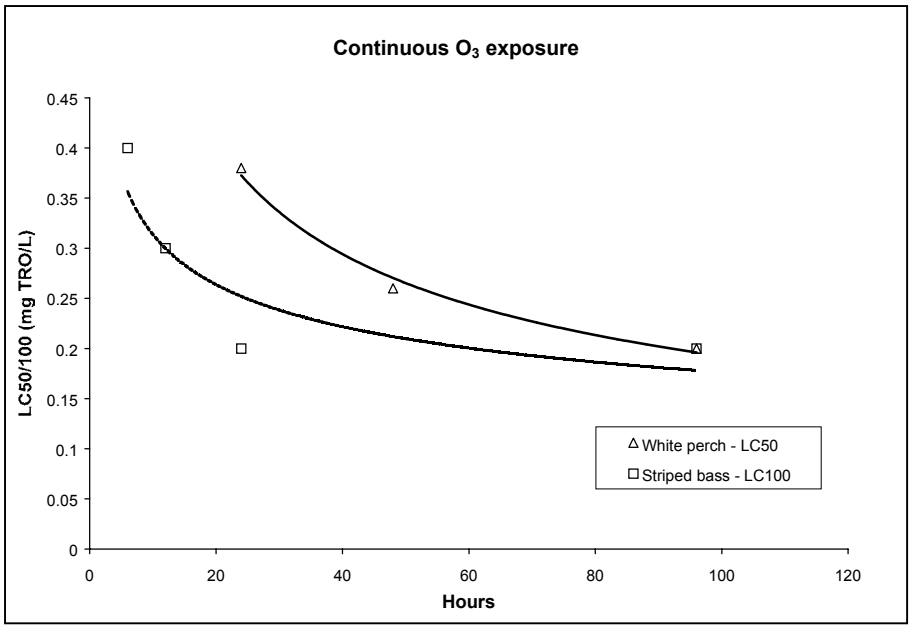


Figure 3.3.1: Toxicity of TRO to striped bass and white perch over time. Curves represent power functions fit to the data by species.

Like most contaminants, ozone toxicity is likely to increase as a function of increasing exposure time, although supporting data are scarce. While even short exposures (e.g., 5 minutes)

were sufficient to induce significant mortality in some organisms (Table 3.3.1), relatively high concentrations (e.g., 1 mg TRO/L) were sometimes required to induce this effect. The effect of time on ozone toxicity is perhaps best observed in 96 hours continuous exposures in which mortality was observed at various times throughout the tests. In experiments with striped bass fingerlings continuously exposed to ozone up to 96 hours, LC100 values decreased by 50% at 24-96 hours compared to that observed at 6 hours (Table 3.3.1; Figure 3.3.1). A similar relationship was observed for white perch adults, but using LC50 values rather than LC100 values with striped bass (Figure 3.3.1). Thus, ozone may be highly effective either via high concentration short-term exposures (e.g., 1 mg TRO/L for 5 minutes for microalgae), or via low concentration long-term exposures (e.g., 0.2 mg TRO/L for 96 hours for striped bass or white perch).

Given that TRO likely consists of bromine species in seawater (Section 7.3; Crecelius 1979), one would expect that bromine toxicity would be similar to ozone-generated TRO. Although the data are sparse, and most of that is for freshwater species, the literature confirms this expectation with LC50 values for fishes and invertebrates ranging from 0.015 – 1.5 mg bromine/L (Table 3.3.2). This further suggests that bromine may be the dominant ozone-produced residual oxidant of toxicological importance in seawater. Alternatively, bromine may be the only effective ozone-produced oxidant that persists long enough to have been measured in the toxicity tests conducted to date.

The most stable by-products of seawater ozonation typically are bromate ion and bromoform and both may persist long after ozone treatment is terminated (Section 3.2). However, the limited available toxicity data set suggests that these compounds are not acutely toxic with LC50 values 1 – 2 orders of magnitude higher than either TRO or bromine (Tables 3.3.3, 3.3.4). The most sensitive species to bromate ion is the mysid shrimp *Neomysis awatschensis* with an acute LC50 of 176 mg bromate ion/L, and the most sensitive species to bromoform is the sheepshead minnow with 96-hours LC50 values ranging from 7.1 – 18 mg bromoform/L. Therefore, even if bromate ion and/or bromoform are produced as by-products of seawater ozonation, they are not likely to be of toxicological concern (Section 7.3.4; see also Crecelius 1979).

Table 3.3.1. Toxicity of ozone to marine organisms (except as noted).

Species	Endpoint	Effect	Test type	Duration	TRO (mg/L)	Exposure Time	Notes	Reference
PHYTOPLANKTON								
Nannochloris sp.		growth / biomass	static	24 hr	0.45	10-15 min	severe biomass decrease in 24 hr after 10-15 min exposure	Toner and Brooks 1975
Nannochloris sp.		growth / biomass	static	72 hr	0.45	5 min	severe biomass decrease in ca. 72 hr after 5 min exposure, then recovers	Toner and Brooks 1975
Monochrysis lutheri		growth / biomass	static	48 hr	1.0	5 min	severe biomass decrease in ca. 48 hr after 5 min exposure	Toner and Brooks 1975
Skeletonema costatum		growth / biomass	static	24 hr	0.10	5 min	complete biomass depletion in 24 hr after 5 min exposure	Toner and Brooks 1975
INVERTEBRATES								
Homarus americanus (American lobster)		survival		6 d	0.40		no effect noted in larval survival or development to 2nd stage	Moffett and Shleser 1975
Crassostrea virginica (oyster)		"stress"	flow	1/2 d	1.0		no discernable adverse effect, in fact, it "improved handling characteristics"	Ciambrone 1975
Crab (species not identified)	0-20%	mortality		24 hr	0.080	1 min	zoea	Toner and Brooks 1975
Crab	0-20%	mortality		24 hr	0.080	1.5 min	zoea	Toner and Brooks 1975

Species	Endpoint	Effect	Test type	Duration	TRO (mg/L)	Exposure Time	Notes	Reference
Crab	40-70%	mortality		24 hr	0.14	5 min	zoea	Toner and Brooks 1975
Crab	100%	mortality		24 hr	0.329	10 min	zoea	Toner and Brooks 1975
Crab	30-40%	mortality		48 hr	0.080	1 min	zoea	Toner and Brooks 1975
Crab	50%	mortality		48 hr	0.080	1.5 min	zoea	Toner and Brooks 1975
Crab	80-90%	mortality		48 hr	0.140	5 min	zoea	Toner and Brooks 1975
Crab	100%	mortality		24 hr	0.20	1 min	megalops	Toner and Brooks 1975
Crab	100%	mortality		24 hr	0.20	2 min	megalops	Toner and Brooks 1975
FISHES								
Morone saxatilis (striped bass)	LC50[a]	mortality	flow	12 hr	0.21	continuous	eggs - estuarine water	Hall et al. 1981
Morone saxatilis	LC50	mortality	flow	30 hr	0.21	continuous	eggs - estuarine water	Hall et al. 1981
Morone saxatilis	LC50	mortality	flow	12 hr	0.060	continuous	eggs - freshwater	Hall et al. 1981
Morone saxatilis	LC50	mortality	flow	30 hr	0.060	continuous	eggs - freshwater	Hall et al. 1981
Morone saxatilis	LC50	mortality	flow	6 hr	0.15	continuous	larvae - estuarine water	Hall et al. 1981
Morone saxatilis	LC50	mortality	flow	96 hr	0.080	continuous	larvae - estuarine water	Hall et al. 1981
Morone saxatilis	LC50	mortality	flow	6 hr	0.20	continuous	fingerlings - estuarine water	Hall et al. 1981
Morone saxatilis	LC50	mortality	flow	96 hr	0.080	continuous	fingerlings - estuarine water	Hall et al. 1981
Morone saxatilis (larvae)	LC100[b]	mortality	flow	6 - 96 hr	0.15	continuous	larvae - estuarine water. LC100 = lowest concentration where complete mortality observed (no stats)	Hall et al. 1981

Morone saxatilis (fingerling)	LC100	mortality	flow	6hr	0.40	continuous	fingerlings - estuarine water	Hall et al. 1981
Morone saxatilis (fingerling)	LC100	mortality	flow	12 hr	0.30	continuous	fingerlings - estuarine water	Hall et al. 1981
Morone saxatilis (fingerling)	LC100	mortality	flow	24-96 hr	0.20	continuous	fingerlings - estuarine water	Hall et al. 1981
Morone americana (white perch)	LC100	mortality	flow	6 hr	0.80	continuous		Block et al. 1978
Morone americana	LOEC[c]	blood pH	flow	1 hr	0.80	continuous		Block et al. 1978
Morone americana	LOEC	blood pOsm	flow	3 hr	0.80	continuous		Block et al. 1978
Morone americana	LOEC	blood pCl	flow	3 hr	0.80	continuous		Block et al. 1978
Morone americana	LOEC	blood pK	flow	2 hr	0.80	continuous		Block et al. 1978
Morone americana	LOEC	blood pMg	flow	1 hr	0.80	continuous		Block et al. 1978
Morone americana	LOEC	blood pCa	flow	1 hr	0.80	continuous		Block et al. 1978
Morone americana	LOEC	gill protein	flow	5 hr	0.80	continuous		Block et al. 1978
Morone americana	LC50	mortality	flow	24 hr	0.38	continuous		Richardson et al. 1983
Morone americana	LC50	mortality	flow	48 hr	0.26	continuous		Richardson et al. 1983
Morone americana	LC50	mortality	flow	96 hr	0.20	continuous		Richardson et al. 1983
Menidia menidia (silverside minnow)		100% mortality		30 min	0.14	5 min	25% control mortality after 24 hr	Toner and Brooks 1975

[a]LC50 = 50% lethal concentration. Concentration of chemical that causes 50% mortality in an acute toxicity test.
[b]LC100 = 100% lethal concentration. Concentration of chemical that causes 100% mortality in an acute toxicity test.
[c]LOEC = Lowest observed effect concentration. Lowest exposure concentration which a statistically significant adverse effect was observed.

Table 3.3.2. Toxicity of bromine to aquatic organisms.

Species	Endpoint	Effect	Test type	Duration	Conc. (mg/L)	Reference
INVERTEBRATES						
Daphnia magna (water flea)	LC50[a]	mortality	static	24 hr	1.5	LeBlanc 1980
Daphnia magna	LC50	mortality	static	48 hr	1	LeBlanc 1980
FISHES						
Lepomis macrochirus (bluegill sunfish)	LC50	mortality	static	24 hr	0.52	USEPA 1995a
Oncorhynchus mykiss (rainbow trout)	LC50	mortality	static	24 hr	0.31	USEPA 1995a
[1] *Strongylocentrotus droebachiensis* (green sea urchin)	EC50[b]	reproduction	static	5 hr	0.015	Dinnel et al. 1981

[a]LC50 = 50% lethal concentration.
[b]EC50 = 50% effect concentration. Concentration which an absolute test endpoint value is 50% of the absolute value in the controls.
[1] Marine species

Table 3.3.3. Toxicity of bromate ion to marine organisms.

Species	Endpoint	Effect	Test type	Duration	Bromate (mg/L)	Reference
INVERTEBRATES						
Crassostrea gigas (oyster)	EC50[a]	develop-ment	static	2 d	30	Crecelius 1979
Neomysis awatschensis (mysid shrimp)	LC50[b]	mortality	static	1 d	176	Crecelius 1979
Macoma inquinata (bentnosed clam)	LC100[c]	mortality	static	3 d	880	Crecelius 1979
Pandalus danae (connstripe shrimp)	LC100	mortality	static	3 d	880	Crecelius 1979
Protothaca staminea, (littleneck clam)	LC100	mortality	static	3 d	880	Crecelius 1979
FISHES						
Oncorhynchus keta (chum salmon)	LC50	mortality	static	4d	512	Crecelius 1979
Cymatogaster aggregata, (shiner perch)	LC100	mortality	static	3d	880	Crecelius 1979

[a]EC50 = 50% effect concentration. Concentration which an absolute test endpoint value is 50% of the absolute value in the controls.
[b]LC50 = 50% lethal concentration.
[c]LC100 = 100% lethal concentration.

Table 3.3.4. Toxicity of bromoform to marine organisms.

Species	Endpoint	Effect	Test type	Duration	Bromoform (mg/L)	Reference
Phytoplankton						
Skeletonema costatum	IC50[a]	growth	static?	7 d	32	Erickson and Freeman 1978
Thalassiosira pseudonana	IC50	growth	static?	7 d	32	Erickson and Freeman 1978
Glenodinium halli	IC50	growth	static?	7 d	32	Erickson and Freeman 1978
Isochrysis galbana	IC50	growth	static?	7 d	32	Erickson and Freeman 1978
INVERTEBRATES						
Americamysis bahia (mysid shrimp)	LC50[b]	mortality	flow	4 d	24.4	USEPA 1978
Penaeus aztecus (brown shrimp)	LC50	mortality	flow	4 d	26	Anderson et al. 1979
FISHES						
Brevoortia tyrannus (Atlantic menhaden)	LC50	mortality	flow	4 d	12	Anderson et al. 1979
Cyprinodon variegatus (sheepshead minnow)	LC50	mortality	static	1 d	19	Heitmuller et al. 1981
Cyprinodon variegatus	LC50	mortality	static	2 d	19	Heitmuller et al. 1981
Cyprinodon variegatus	LC50	mortality	static	3 d	18	Heitmuller et al. 1981
Cyprinodon variegatus	LC50	mortality	static	4d	18	Heitmuller et al. 1981
Cyprinodon variegatus	LC50	mortality	flow	4d	7.1	Ward et al. 1981
Cyprinodon variegatus	NOEC[c]	juv.mort.	flow	28 d	4.8	Ward et al. 1981
Cyprinodon variegatus	LOEC[d]	juv.mort.	flow	28 d	8.5	Ward et al. 1981

[a]IC50 = 50% inhibition concentration. Concentration which a test endpoint is inhibited by 50% compared to controls.
[b]LC50 = 50% lethal concentration.
[c]NOEC = No observed effect concentration.
[d]LOEC = Lowest observed effect concentration.

4 EXPERIMENTAL SYSTEM

4.1 The *S/T Tonsina*

The *S/T Tonsina* is an 869-foot American-flagged oil tanker operated by Oregon-based Alaska Tanker Company in what is commonly known as the TAPS (Trans Alaskan Pipeline Service) trade of Alaska North Slope crude oil. This oil is transported mainly between Valdez, Alaska and refineries on the west coast of the United States. The *S/T Tonsina* can carry 270,000 barrels of ballast water, or more than 11 million gallons (41,600,000 L) in its 12 ballast water tanks, and 807,000 barrels (nearly 34 million gallons) of crude oil in its 12 cargo tanks.

The *S/T Tonsina* has a double hull, which means that the cargo tanks are protected by an outer hull, and the space between the hulls is divided transversely into segregated sections for carrying ballast water when the ship is empty or only partially loaded. These ballast tanks are arranged along the vessels' outer hull and double bottom area (see Figure 4.1.1). Although each wing tank area is connected to the double bottom tank area, water circulation between these two areas is believed to be poor.

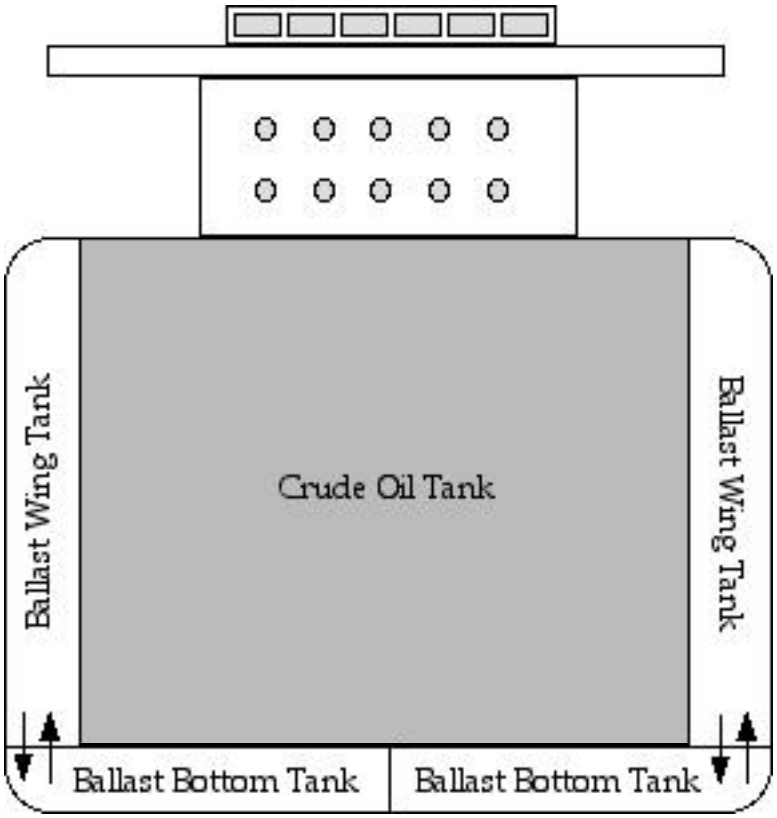


Figure 4.1.1. Cross section (not to scale) of the *S/T Tonsina's* ballast tank arrangement. The arrows signify the presence of some circulation between the wing and bottom tanks, but the extent of this circulation is believed to be limited.

4.2 The Ozone System

In the fall of 2000, a prototype Nutech ozonation system was installed on the *S/T Tonsina* during a planned out-of-service period, while afloat at the Hyundai Mipo Drydocks in Ulsan, South Korea. The customized prototype, known as the SCX 2000, was built to fit inside a standard ISO 20 foot container in order to facilitate the installation. The container was installed on the *S/T Tonsina's* stack deck, in an exterior location.

Ozone is produced by sending a stream of oxygen-enriched compressed air through a series of water-cooled electrodes. Within each electrode, a high voltage corona discharge is created (an electric arc), using a standard ship's 480-volt power transformed to more than 10,000 volts. As the oxygen enriched air stream passes through each corona gap, a percentage of the air stream is converted into ozone, which is then collected and piped into each of the 12 ballast tanks, through a system of flow meters and stainless steel pipe. The ozone is distributed throughout each ballast tank by a system of 1,200 custom designed ceramic coated stone diffusers, arranged to maximize the distribution and contact time of the ozone. Ballast tanks can be ozonated individually or in groups, with the prototype's maximum system capacity of 1800 gm O_3/hour, leading to an O_3 loading rate of ca. 0.6 mg/L/hour in each tank when treated individually.

Because the *S/T Tonsina* is double-hulled, its ballast tanks are between the hulls that surround the ship's central oil cargo tanks and are separated into a series of baffled chambers. The chambers are interconnected vertically and horizontally by openings large enough for maintenance personnel to pass through. At the top of each series of chambers is either a manhole for personnel access or an approximately 12-inch Butterworth® hatch used for the deployment of cleaning equipment (Figure 4.2.1).

The ozone gas diffusers were arranged in 8 rows running horizontally with the beam of the vessel, with 7 rows placed in the double bottom section of the ballast tank (underneath the oil cargo tank), and 1 row placed at the bottom of the vertical side tank (Figure 4.2.2), in the curve of the bilge area.

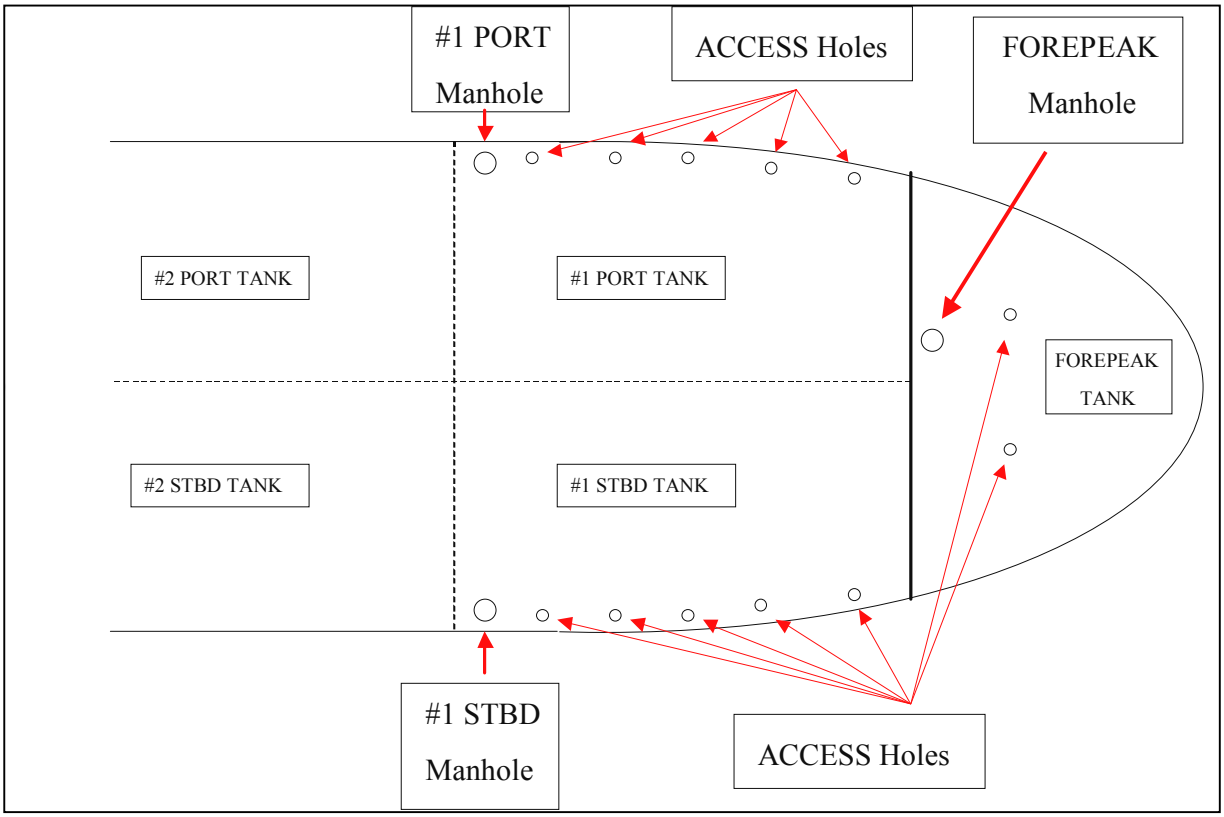


Figure 4.2.1. Top view of access hatches on deck of S/T *Tonsina*

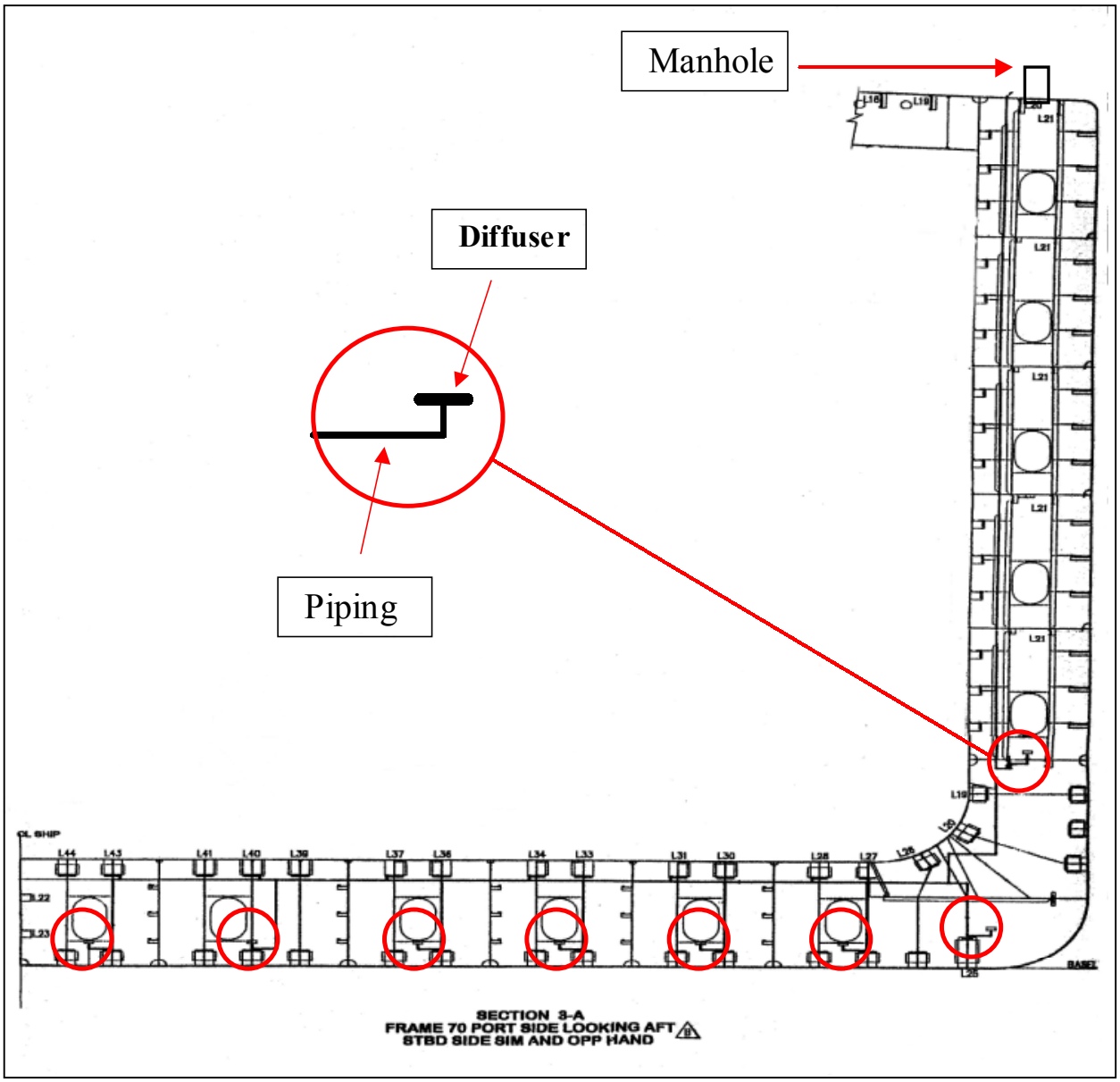


Figure 4.2.2. Layout of ozone diffusers shown in cross section of the ballast tanks on the *S/T Tonsina.*

5 PRELIMINARY STUDIES

Preliminary studies of this system were undertaken in November 2000 on board the *S/T Tonsina*. The experimental design was relatively simple. The two No. 3 ballast wing tanks (port and starboard) were studied. The port was the control to which no ozone was added and the starboard was ozonated. Samples were withdrawn immediately prior to the initiation of the test (at 0 hours) and then at 2, 4, 6, 8 and 12 hours after ozonation or oxygen bubbling (for the control tank). There were 10 sample lines in each tank. The samples were drawn from several depths in each tank (Figure 5.1.1). In addition to bacterial counts, bromate and bromoform concentrations were measured.

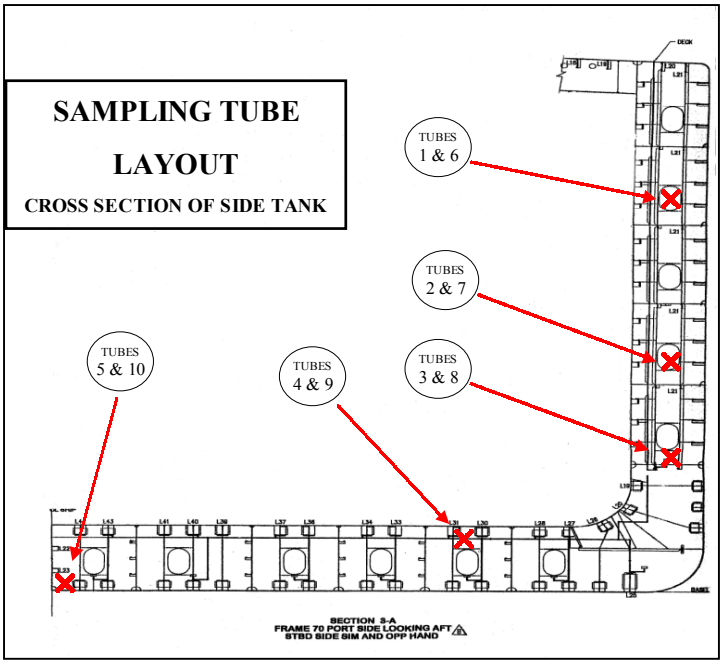


Figure 5.1.1. Sample Tube Locations

The results are summarized below for the bacterial kill (Table 5.1.1) and bromoform formation (Table 5.1.2). There was no bromate ion found (detection limit of 1 μg/L) in any of the samples.

Table 5.1.1. Summary of the bacterial numbers (direct count) in the ozone treated ballast tank, 3S.

Sample Line	Time (hours)					
	0	2	4	6	8	12
1	6.9×10^4	1.9×10^4	2.3×10^3	3.6×10^2	4.4×10^2	5.6×10^2
2	1.1×10^5	3.6×10^3	7.8×10^2	5.1×10^2	8.5×10^2	3.3×10^2
3	3.1×10^3	5.3×10^3	1.5×10^3	3.5×10^2	2.0×10^2	1.5×10^2
4	4.3×10^3	1.2×10^3	1.5×10^2	1.5×10^2	3.6×10^1	3.6×10^1
5	3.7×10^3	5.6×10^2	1.3×10^2	9.1×10^1	9.1×10^1	9.1×10^1
6	6.4×10^3	2.6×10^3	7.7×10^2	5.4×10^2	4.1×10^2	3.2×10^2
7	5.2×10^4	2.1×10^3	5.0×10^2	1.8×10^2	4.4×10^2	3.6×10^2
8	9.3×10^3	4.7×10^2	2.4×10^2	9.3×10^2	6.4×10^2	1.8×10^2
9	1.4×10^4	2.3×10^4	1.5×10^3	3.6×10^2	5.0×10^2	3.6×10^2
10	6.9×10^4	3.8×10^4	1.5×10^3	3.3×10^2	1.1×10^2	1.6×10^2

Table 5.1.2. Summary of the bromoform concentration (µg/L) in the ozone treated ballast tank, 3S.						
Sample Line	Time (hours)					
	0	2	4	6	8	12
1	BMDL[1]	11.2	NA[2]	117.9	174.9	171.6
2	BMDL	112.8	NA	148.8	89.8	NA
3	BMDL	53.3	111.1	89.6	172	158.5
4	BMDL	47	112.2	121.3	147.4	164.2
5	BMDL	83.9	133.7	161.3	321.8	255.5
6	BMDL	61.1	138.9	137.4	154.4	207.8
7	BMDL	130.5	153.4	145.4	179.1	215.1
8	BMDL	112	142.3	178.7	177.9	294.2
9	BMDL	42.3	98.6	115.4	161.6	168.4
10	BMDL	9.6	NA	NA	NA	177.2
1 BMDL = below method detection limit 2 NA = not analyzed						

A summary of the relationship of bromoform formation and bacterial kill is shown (plotted as arithmetic means for all 10 sample lines) in Figure 5.1.2.

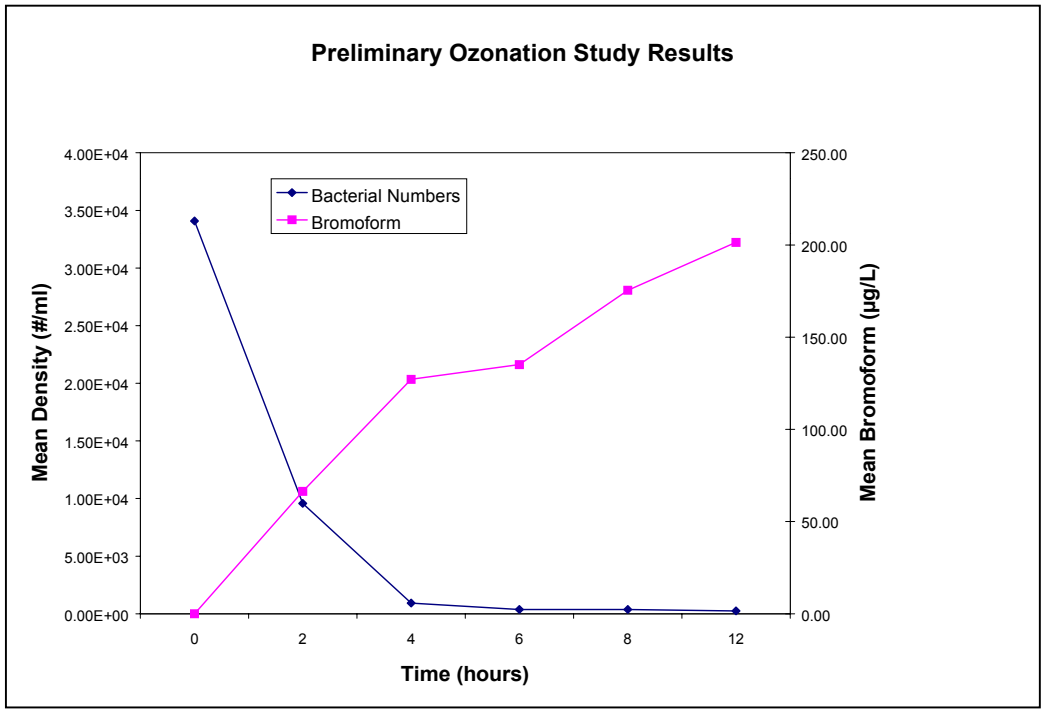


Figure 5.1.2. Plot of bacterial numbers and bromoform concentrations over time

The results showed that the *S/T Tonsina's* ozone treatment system achieved, on average, 99% removal of the bacteria after four to six hours. The bromoform concentration varied somewhat but was in general slightly higher than 100 µg/L at the time 99 % bacterial removal

was observed. These data suggest that ozonation was highly effective in removal of free-living bacterial concentrations. While this occurred simultaneously with the generation of bromoform as a reaction by-product, it is doubtful that these bromoform concentrations would be high enough to cause a direct toxic impact. Rather, it is likely that ozone and its reactants (Table 3.2.1 and 3.2.2) may have been most responsible for reductions in bacterial numbers.

6 MATERIALS AND METHODS

6.1 Overview

On four occasions, the 13-member experimental team assembled to conduct ozone experiments aboard the *S/T Tonsina* while she was anchored at Port Angeles in Washington State. This occurred once in May, once in September and twice in November of 2001. In May, the ozone experiment was terminated after less than two hours of ozonation because the electrical transformers that provide power to the ozone generator began to overheat and had to be shut down to prevent further damage to the system. Although this prevented the collection of any ozone-related data, the experimental team elected to follow through as scheduled with the BWE portion of the experiment during the return voyage to Valdez, Alaska. It took several months before the ozone generator was repaired and the system was again available for testing.

On September 24, a five-hour ozonation experiment was successfully conducted (Experiment 1). Following review of the data from that experiment, the team decided to change the exposure period to 10 hours for the final two experiments, which were held on November 2 (Experiment 2) and November 4 (Experiment 3). The second and final BWE experiment was conducted in September following the first successful ozone experiment. BWE experiments were not conducted following the second and third ozone experiments in November because the *S/T Tonsina* did not sail into open ocean following these experiments but rather sailed to Portland, Oregon, for repairs and temporary lay-up.

In all three-ozone experiments, the No. 3 port wing ballast tank was used for the ozone treatment tank, and the No. 3 starboard wing ballast tank was used for the control tank. These are both tall, vertically oriented tanks that were sampled from several access points on the ship's deck. Five-liter Niskin bottles were used to collect water samples at three depths from these tanks, and sub samples for chemistry, bacteria and phytoplankton were collected from these bottles and processed into the appropriate containers and were analyzed using methods described below. A zooplankton net was used to collect zooplankton samples from the tank, and these samples were immediately examined under a microscope on board the ship. Caged organisms were also deployed at four depths in both tanks and the kill ratio was established by determining the live/dead/moribund status following the ozonation exposure period as described below.

Each sample type was collected from two access points above each tank to help assess some amount of spatial variability in ozone system efficacy. Each tank was sampled in two sections (i.e.,columns), with the forward and rear portion of the treatment tank referred to as Column A and Column B, respectively, and the forward and rear portion of the control tank referred to as Column C and Column D (See Figure 6.1.1). Since Niskin bottle samples were collected from three depths at each column at each time point, this gave rise to a letter/number sample labeling where the letter signified a time point (T) or column location (A, B, C, D) and the number signifies either the time or depth (in feet) from the surface at which it was taken. For example, sample T-0.0B50 represents the sample at the initial time (T) of the experiment (0.0), before the ozone generator was engaged, and the column (B) and depth (50 feet) at which it was taken. Another example would be T-7.5A10, which would represent the sample time of 7.5 hours from the beginning of ozone inundation at the depth 10 feet from the surface in Column A.

In Experiment 2 and Experiment 3, Column D was dropped due to the increased samples collected with additional time points. Column C then became the only control tank column and was moved from the front to the middle of the tank.

As each filled Niskin bottle was brought to the surface, a single water quality sample was collected to measure pH, salinity, dissolved oxygen, and oxidation-reduction potential. Duplicate samples were then collected into specimen cups for immediate analysis of both ozone and TRO (Section 6.3.2). Ozone Accu-vacs were used for the ozone spectrometric analysis and total chlorine Accu-vacs were used for TRO analysis. These analyses were made using a portable analysis kit (DREL with a DR/2010 spectrometer manufactured by the Hach Company, Loveland, CO), which was set up on board the tanker.

From these same Niskin casts, samples were collected and immediately placed on ice for later transport to the respective analytical laboratories: samples for bacteria analysis were collected into one-liter polypropylene bottles, samples for phytoplankton analysis were collected into one-liter HDPE bottles, samples for bromate ion analysis were collected in 50 ml HDPE bottles, and duplicate samples for bromoform were collected in 40 mL glass amber VOA vials (Section 6.3).

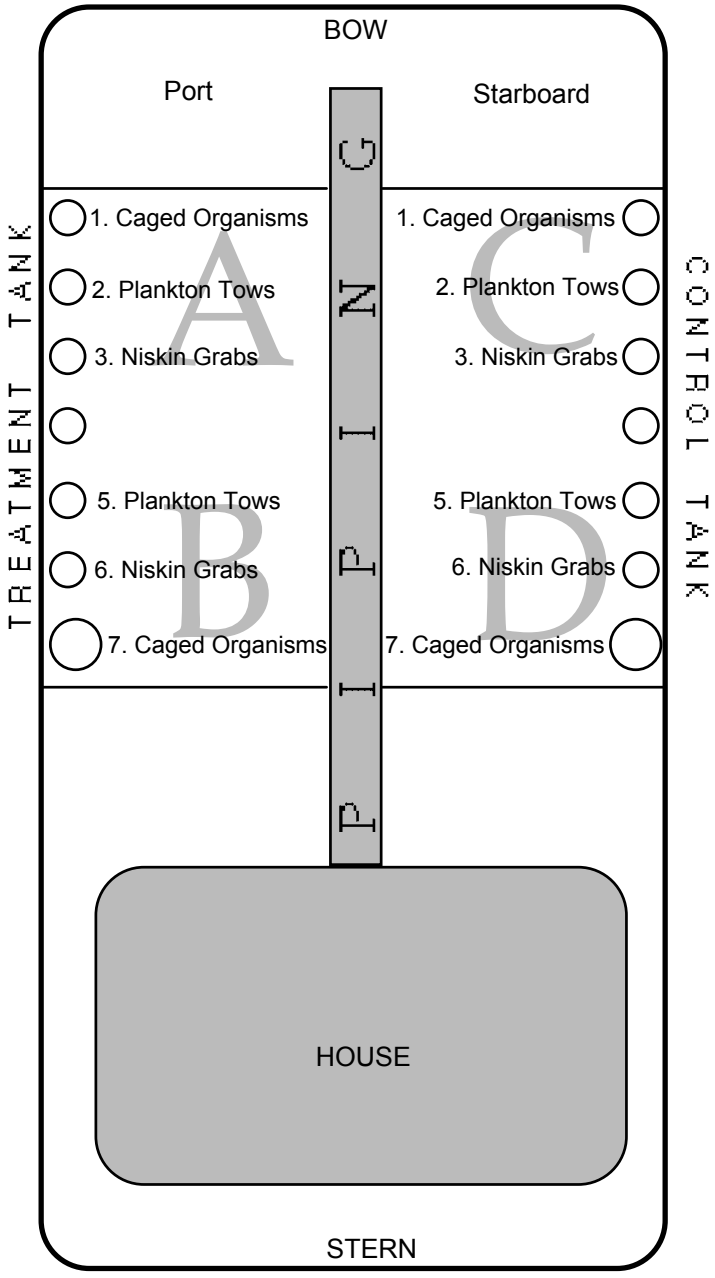


Figure 6.1.1. Schematic of the layout of No. 3 port and starboard ballast tanks on deck. The seven access points to each wing tank are shown, along with corresponding samples types. Drawing is not to scale, as there are also several other such ballast wing tanks on each side of the ship. Note that Column D was not sampled in Experiments 2 and 3.

6.2 Ozone Delivery

The ozone system installed on the *S/T Tonsina* is capable of delivering ozone to each vertical wing tank and horizontal bottom tank, even though these are interconnected, making the two tanks essentially two sections of one L-shaped tank. However, as mentioned earlier, circulation between these two sections is believed to be poor, thus, results are likely to occur as if they were completely separate tanks with respect to the movement of water or organisms.

The ability to distribute ozone between the two sections was examined in the three experiments. Although this diminished replication between experiments, varying the ozone delivery revealed how the chemistry and biology in the tank responded to different ozone-loading rates.

In Experiment 1, ozone delivery between the vertical and horizontal section was evenly divided, with 50% going to each section. In Experiment 2, 60 percent of the ozone was delivered to the vertical section (which was the section sampled during the experiments) with the remaining 40% delivered to the bottom horizontal section. In Experiment 3, 100 percent of the ozone was delivered to the vertical section and none to the horizontal section. The most effective biological kill ratios were seen in this last experiment.

6.3 Water Chemistry

6.3.1 Water Quality

Water quality analyses were conducted on board ship using a Hach DREL/2010 Water Quality Laboratory and nutrients were later analyzed in a laboratory. All water quality samples were obtained from Niskin bottle grabs, and analyzed according to the instructions provided with the Hach water quality test kit. The determination of pH was conducted using a Hach Portable pH Meter (Hach Company, Loveland, CO). Dissolved oxygen was measured with a model 21800-022 Traceable® digital dissolved oxygen meter. This meter was air calibrated and adjusted to compensate for salinity. Salinity was measured using a conductivity meter with a range of 0-80 ‰ (Hach Company, Loveland, CO). Temperature was determined using a standard field thermometer. Inorganic nutrients (ortho-phosphate, nitrite, nitrate, ammonia, silicic acid) were analyzed from stored, refrigerated samples at the University of Washington using standard colorimetric techniques.

6.3.2 Ozone Chemistry

Total Residual Oxidant (TRO): TRO was determined using a standard DPD colorimetric analysis for total chlorine (APHA 1998). Samples were collected and KI was added and the TRO determined spectrophotometrically in the range 0-4.5 mg/L as Cl_2. This was achieved by using Hach® brand Accu-vac vacuum reaction containers, which were submerged and filled with ballast water samples immediately after the samples were collected from the tank. The filled Accu-vac containers were analyzed on a Hach® DREL 2010 spectrometer water quality lab kit.

Ozone: The presence of ozone was measured using an Indigo colorimetric technique (APHA 1998). Similar to TRO, Accu-vacs reaction containers were used with fresh Niskin grab samples and analyzed by using a DREL 2010 kit.

Oxidation Reduction Potential (ORP): Oxidation-reduction potential was measured by using an Orion 290A pH meter with a Cole-Palmer Combination ORP probe (Pt electrode, Ag/AgCl reference cell). In Experiments 2 and 3, additional ORP measurements were read directly inside ballast water tanks using a Hydrolab (Austin, TX) Quanta monitoring system that included an Ag/AgCl ORP sensor (see Section 7.7.4).

Bromate ion: Samples for bromate ion analysis were collected in 150 mL wide-mouth HDPE bottles. These were stored on ice and shipped to analytical laboratories as soon as possible after completion of the study. Two ion chromatography methods are available for measuring bromate ion: USEPA Method 300.1 and USEPA Method 317. Method 300.1 employs conductivity detection while Method 317 uses a post-column detection procedure to overcome interferences that may occur using Method 300.1. For seawater with higher ionic strength, when the chloride ion can potentially interfere in Method 300.1, Method 317 (EPA Document # EPA 815-B-01-001; http://www.epa.gov/safewater/methods/sourcalt.html) was used.

These methods incorporate chlorpromazine reaction chemistry to measure low-level bromate ions (BrO_3^-) following the separation of anions. The post-column procedure uses photometric detection at 530 nm (nanometers). Following separation, BrO_3^- reacts with chlor-promazine in acidic media by a charge transfer mechanism. This method overcomes well-known problems encountered in the ion chromatographic measurement of BrO_3^- from Cl^- and CO_3^{2-}. This procedure shows no interference from all common anions with the exception of chlorite ion and nitrite ion. This method can be used to measure bromate ion from 3 to 40 µg/L. The method detection limit (MDL) was determined to be 2-3 µg/L bromate ion.

For Experiment 1, the samples were shown to have a bromate ion concentration less than 2 µg/L. During the first set of analyses, it was observed that QA samples spiked with bromate ion were "unrecoverable." The subsequent evaluation of bromate ion standards prepared in distilled water (i.e., standards) showed good recovery.

Thus, studies were conducted to ascertain why spiked bromate ion in the ballast water samples could not be recovered. These studies showed that at higher concentrations (i.e., at the mg/L level), spiked bromate ion could be recovered. Subsequently, all of the ballast water samples were diluted to 20 % of their original concentration (1:5 dilution). With this dilution, it was determined that adequate bromate ion recovery could be achieved at the 50 ppb level.

Based on the bromate ion recovery following a 1:5 dilution, all of the ballast water samples for experiments 2 and 3 were diluted. Because the detection limit of the method is approximately 2 µg/L bromate ion, this dilution would still enable the detection of 10 µg/L bromate ion, which is the MCL established for bromate ion in drinking water.

Bromoform: Samples for bromoform analysis were collected in 40-mL VOA vials and were stored on ice and shipped to the analytical laboratory as soon as possible after completion of the study. The maximum acceptable sample analysis holding time is 14 days after sample collection. Bromoform was analyzed using a purge and trap (dynamic stripping) system coupled to a Hewlett Packard Model 5890 Series II gas chromatograph. The chromatograph was equipped with a 30 meter VOCOL capillary column, HP 3396A integrator/printer, and flame ionization detector. Bromoform was obtained from Ultra Scientific (product #HC-020, 100 ng/µL $CHBr_3$, Lot # R-1194 Standard Reference Material (SRM) traceable to the National Institute for Standards and Testing). Tekmar Model LSC-2000 Liquid Sample Concentrator interfaced with a Tekmar Model 2016 Autosampler system. Ultra pure Carrier-grade helium gas was used for sparging samples.

Initial calibration and calibration verification checks were preformed using known amounts of SRM prepared within laboratory-purified water. The standards used were with concentrations of 5, 10, 20, 50, 100 and 200 µg/L. Each solution was analyzed and the average instrument response factor was calculated by dividing the area counts observed for each standard solution.

Quantities of 5.0 ml were sub-sampled from the field sample bottle by using a gas tight syringe, after 200 ng of surrogate standard, a,a,a-trifluorotoluene was added to the sub-samples. Samples were sparged with helium gas for 12 minutes at a rate of 30 mL/minutes onto a Tenax trap at ambient temperature (less than 25 ˚C). After completion of the sparge cycle, the sample was desorbed from the Tenax trap at 250 ˚C for two minutes. The sample was transferred to the gas chromatograph splitless inlet using a heated nickel transfer line. After the transfer was completed, the Tenax trap was baked at greater than 250 ˚C for eight minutes between samples. The chromatogram was recorded on the HP 3396A integrator by setting it at the proper sensitivity to produce peak height of the surrogate compound to greater than 50% full scale. The sample location, date, time and sample volume were recorded for each analysis on the integrator printout and within the bound laboratory GC Logbook.

Initial GC external standard calibrations were conducted by preparing a multipoint instrument calibration by injecting a range of volumes of the $CHBr_3$ SRM into 5 mL of laboratory purified water. Each solution was analyzed and a calibration curve plotted. A linear regression coefficient for the SRM concentrations was determined. If the regression coefficient was greater than 0.997, the calibration was acceptable for the range of concentrations analyzed.

A calibration verification check sample (CVCS) with an SRM concentration equivalent to approximately 50% of the highest standard solution was analyzed twice each day whenever process samples were analyzed: once prior to the first process sample analysis, and once at the end of the day's analytical batch. The percent recovery was calculated by dividing the actual concentrations of bromoform detected by the theoretical concentration of the CVCS standard analyzed and multiplying by 100 %. If the calculated recovery was below 50% or greater than 150%, the CVCS standard for that analytical batch was unacceptable and the CVCS was reanalyzed and a new calibration curve was determined.

Two calibration verification standards were analyzed per day. The percent recovery of each compound was calculated and recorded on the quality control chart. A sample was analyzed in duplicate once per week and the relative percent difference (RPD) for the detected concentration in the process sample was determined. This was achieved by dividing the range of the detected concentrations by the mean of concentrations and multiplying by 100 %. A RPD of less than 30 % was considered to be acceptable. In addition, all method blank analyses with each batch of process samples were preformed. Target bromoform detections equal to or greater than two times the method detection limit were considered non-compliant. For non-compliant tests, appropriate corrective action was performed and each affected analysis was repeated.

6.4 BWE Experiments

The exchange experiments were conducted using *S/T Tonsina* protocols for open ocean exchange. A simpler version of the ozonation experimental design was used in this experiment. Only a limited crew (two or three) rode the *S/T Tonsina* on her return to Valdez carrying the same ballast water as was used in the ozonation experiment. This was done once in May following an aborted ozonation experiment (due to electrical problems with the ozone generator), and once in September following the five-hour ozonation experiment. Niskin grab samples were collected for simple water chemistry (e.g., pH, DO, nutrients) and microbial and plankton community composition were determined. Net tow samples provided organisms for the zooplantkton analysis.

The sampling design is summarized in Table 6.4.1. Both of these experiments used the same control tank as the ozone experiments, the No. 3 starboard wing ballast tank. The treatment tank that underwent BWE was the No. 4 port wing ballast tank, a tank adjacent and nearly identical to the ozone treatment tank (No. 3 port). For each of the two ballast exchange experiments, the BWE tank (No. 4 port wing) was sampled prior to the ozone experiment, prior to the exchange experiment, and after the exchange experiment. The type of exchange for the May experiment was a 100 % empty/refill, while the September experiment was 200 % empty/refill.

Both the control and exchange tanks were sampled with a zooplankton net identical to that used in the ozone experiments, and these samples were fixed for later microscopic analysis. Both tanks were also sampled using a five-liter Niskin bottle that provided sample for phytoplankton and chemistry analysis.

Table 6.4.1. Summary of the sampling schedule for the BWE experiment, with the number of samples *per ballast tank* indicated.

Time	Niskin grabs	Zooplankton Tows
	Phytoplankton	
Pre-ozonation	2 columns x 2 depths x 2 reps. (8)	2 columns x 2 depths (4)
Pre-exchange	8	4
Post-exchange	8	4
Totals	**24**	**12 tows**

6.5 Whole Effluent Toxicity (WET) Testing

Ballast water handling procedures ultimately require the discharge of ballast water into the open-ocean or estuarine waters. "Active" treatment technologies (e.g., addition of chemical sterilants or ozonation) that could result in the formation or introduction of toxic materials in ballast water are likely to come under some degree of regulatory scrutiny to ensure that the discharge of "toxic waters in toxic amounts" (Clean Water Act) does not occur. Vessel operators will likely be required to provide evidence that no adverse effects to organisms in the receiving water will result from the discharge of the treated ballast water. Confirmation of this is likely to be similar to the requirements currently used for monitoring the discharge of permitted point-source effluents via the National Pollutant Discharge Elimination System (NPDES), i.e., a combination of chemical specific measurements and whole effluent toxicity (WET) tests.

In order to see how ozone-treated waters would respond to these tests, samples of ozone-treated ballast waters were submitted for laboratory toxicity testing, using the same methods employed in conducting WET tests. Two standard marine toxicity tests were performed with water samples from post-ozonation ballast-water tanks: 1) the mysid shrimp (*Americamysis bahia*) static acute toxicity test, and 2) the topsmelt (*Atherinops affinis*) static acute toxicity test. These species have been shown to be among the most sensitive organisms when exposed to toxic chemicals in seawater (Suter and Rosen, 1988), and are considered to be suitable surrogates for indigenous species. Both tests are commonly used to evaluate the toxicity of effluents discharged into marine waters.

All toxicity tests were performed in accordance with standard procedures developed by the U.S. Environmental Protection Agency (U.S. EPA 1993, 1999). The seawater used as experimental controls and for dilution of ballast water samples was prepared using laboratory freshwater (1 μm filtered) and commercially available seawater salts (Hawaiian Marine Mix). The seawater strength was 30 ± 2 ‰ salinity. Ballast water samples (from both the ozone-treated and the un-treated control ballast tanks) were collected during each of the three field trials on 24 September 2001 (Experiment 1), 2 November 2001 (Experiment 2), and 4 November 2001 (Experiment 3). Samples were transported as soon as possible (within 24-48 hrs while stored at <4 °C) to the Parametrix, Inc. toxicology laboratory (Kirkland, WA) for testing; all laboratory tests were initiated within 24 hours of sample receipt.

Mysid shrimp were obtained from a commercial supplier (Aquatic Biosystems, Inc., Fort Collins, CO). Mysids (5 days old at the time of test initiation) were exposed for 48 hours in a static test to five dilutions of ballast water: 6.25, 12.5, 25, 50, and 100% ballast water and to a dilution water control. Organisms were maintained at a water temperature of 25 ± 1°C under a 16:8 hour light:dark cycle. Test solutions were not aerated and mysids were not fed during the tests. Four replicate test solutions containing five to ten animals per chamber were used at each treatment level in all tests. Organisms were monitored for survival/mortality daily over the

course of the test and at the end of the test the results were used to determine median lethal concentrations (LC50[2]).

Laboratory test procedures used in conducting the topsmelt test were very similar to those of the mysid tests. Topsmelt larvae (15 days old at the time of test initiation) were obtained from Aquatic Biosystems, Inc. Larvae were exposed for 48 hours in a static test to five dilutions of ballast water samples: 6.25, 12.5, 25, 50, and 100 percent ballast water and to a dilution water control. Five replicate 1-L test chambers, each containing 500 ml of test solution and 5-8 fish, were used at each treatment level in all tests. Organisms were maintained at a water temperature of 25 ± 1°C under a 16:8 hour light:dark cycle. Test solutions were not aerated during the test and larvae were not fed. Organisms were monitored for survival/mortality daily over the course of the test and at the end of the test. The results were used to determine median lethal concentrations (LC50s).

6.6 Bacteria

6.6.1 Culturable Heterotrophic Plate Count

The number of viable heterotrophic bacteria were determined by performing a culture-based microbiological procedure. During the shipboard experiment, ballast water was collected from the ozonated ballast tank and the control ballast tank in 5-L Niskin oceanographic bottles. For enumeration of the microorganisms, a sample from the Niskin bottle was placed in a 1-L sterilized Nalgene bottle. These bottles were placed on ice in a cooler on board the ship, transported to the University of Washington laboratory on ice, and maintained on ice until the samples were processed in the laboratory. Samples were processed at the University of Washington in the Herwig laboratory within 24 hours of collection on board the *S/T Tonsina*. The numbers of culturable heterotrophic bacteria were determined on Marine R2A Agar (Section 6.6.2) by using two methods. Aliquots of ballast water were inoculated onto the surface of the agar by using the spread plate method or a larger volume of seawater was filtered through a membrane filter (Gelman Metricel Black 47-mm diameter, 0.45-µm pore size filters). Filters were placed on the surface of Marine R2A Agar contained in a 50-mm diameter petri plate. Filters were rolled onto the agar surface to prevent air bubbles from forming between the filter and agar. Larger 100-mm diameter petri dishes were used for samples that were inoculated onto the agar by the spread plate method. Samples were generally inoculated in triplicate for each dilution, except for some filtered samples that were inoculated in duplicate. Inoculated media were incubated at room temperature (approximately 22 ^{o}C) in the dark. Bacterial colonies were counted on the spread-plate agar surfaces and membrane filters after 4 days when the colonies were large enough to see but not crowding against one another. The spread plate media were enumerated after 7 days of incubation.

The membrane filtration method was used for the ozonated samples to increase the sensitivity of the assay. A much larger volume of seawater was examined using the membrane filtration method. Filtration was performed with 10 and 100 mL of the sample, and spread plates were inoculated with 100 µL of the original sample or 100 µL from a serial dilution of the

[2] The LC50 represents the concentration of a test material (i.e., ballast water) necessary to kill 50% of a population of exposed organisms

sample. A marine diluent was prepared for the serial dilutions. The formulations for the bacteriological media used in the experiment follows.

6.6.2 Marine R2A Agar

Marine R2A Agar is a modification of a medium that is recommended by EPA for the enumeration of the total number of culturable heterotrophic bacteria in freshwater samples. Marine R2A agar (Table 6.6.1) was supplemented with the salts that are found in seawater. The Herwig Lab has developed a marine salts solution called ONR Seawater Salts (Table 6.6.2, 6.6.3) that contains the major cations and anions found in seawater. For Marine R2A agar, the contents of ONR Seawater Salts replaces distilled water, the liquid that is used to prepare R2A agar. The ONR Seawater Salts solution was prepared as a 10X solution so that 100 mL of the 10X solution is used to prepare 1,000 mL of Marine R2A Agar. The pH of medium was adjusted to 7.6 and the medium was sterilized by autoclaving at 121 ˚C. Following autoclaving, the medium was cooled in a water bath to 50 ˚C. ONR Divalent Cations solution (20.0 mL per liter of 50X solution) and ONR FeCl solution (5.0 mL per liter of 200X solution) were added to the liquid. Divalent cations and Fe were added to the medium after autoclaving to minimize the formation of a precipitate in the medium. The dehydrated form of R2A agar medium is commercially available from Difco (Detroit, MI).

Table 6.6.1. R2A Agar (Difco) constituents.

Yeast Extract	0.5 g
Proteose peptone No.3 or polypeptone	0.5 g
Casamino acids	0.5 g
Glucose (Dextrose)	0.5 g
Soluble Starch	0.5 g
K_2HPO_4	0.3 g
$MgSO_4 * 7H_2O$	0.05 g
Sodium Pyruvate	0.3 g
Agar	15.0 g
Distilled water (ONR Seawater Salts used for Marine R2A)	1,000 ml

Table 6.6.2. ONR Seawater Salts solutions. (1X Concentrations in final Marine R2A Agar preparations)

10X Salts	(g/L)	50X Divalent Cation Salts	(g/L)	200X Fe Salts	(g/L)
NaCl	227.916	$MgCl_2 * 6H_2O$	55.908	$FeCl * 4H_2O$	0.40
Na_2SO_4	39.771	$CaCl_2 * 2H_2O$	7.277		
KCl	7.232	$SrCl_2 * 6H_2O$	0.121		
NaBr	0.833				
$NaHCO_3$	0.309				
H_3BO_3	0.266				
NaF	0.026				

Table 6.6.3. Marine salts, final concentration in R2A Agar

Salt	Per Liter (g)
NaCl	22.792
$MgCl_2 * 6H_2O$	11.182
Na_2SO_4	3.977
$CaCl_2 * 2H_2O$	1.455
KCl	0.723
NaBr	0.083
$NaHCO_3$	0.0309
H_3BO_3	0.0266
$SrCl_2 * 6H_2O$	0.024
NaF	0.0026
$FeCl_2 * 4H_2O$	0.002

Marine Mineral Salts Diluent. ONR Seawater Salts solution with no added carbon source. Dilution blanks containing 9.0 ml were dispensed into 16 x 150 mm screw cap tubes. Autoclave for 15 min at 121 ˚C. Final pH 7.6.

6.6.3 Bacterial Regrowth in Ozonated Ballast Water

To examine the ability of heterotrophic microorganisms to regrow in the period following treatment on board the *S/T Tonsina*, samples were collected from the experimental ballast tank and stored for 35 days. Seawater samples were collected at the end of the 5 hours (T3) and 10 hours (T5) of ozone treatment from the A and B columns of the treated tanks. Five liters of seawater were collected and combined from each ozone treatment and placed in a 10-L sterile Nalgene carboy. A 10-L sample was also collected from the untreated ballast tank at the end of the experiment, which provided for a 10-hour (T5) sample. The 10-L seawater samples were placed on ice until they were returned to the Herwig laboratory at the University of Washington. Here, the carboys were placed inside a 10^{o} C incubator and incubated in the dark for 35 days. Samples were removed from the carboys and inoculated onto the heterotrophic medium as described above. The ozonated seawater sample was concentrated by membrane filtration and the untreated seawater sample was inoculated directly onto the surface of the Marine R2A Agar.

6.7 Zooplankton

A 0.3-m diameter 73-μm mesh zooplankton net was used to collect animals to estimate abundance and condition (mortality or moribund). The net was lowered from two openings in the top of both the control and treatment tank to within 0.25 m of the tank bottom and slowly retrieved to the surface. Three replicate zooplankton vertical hauls were taken from each opening before ozone treatment, and after five hours (all experiments) and ten hours (November experiments) of ozone treatment. Samples were gently washed from the net collecting bucket into a new plastic specimen jar and placed on top of a layer of ice in a bucket. For ozone and control treatments, the samples were immediately examined under a dissecting microscope. A field of view at 25x magnification was examined. Animal activity was scored as follows: if animals were moving of their own accord or moved away when probed with a fine needle (a 000 size insect pin mounted on a wooden stick), they were scored as “alive;” if they were not mobile,

but exhibited internal or external movement, they were scored as "moribund;" and if they showed no life, they were scored as "dead." Successive fields of view were examined until a total of 100 organisms were examined. In addition to these counts, qualitative observations were made about which, if any, taxa appeared to be more or less affected by the treatment.

For the exchange experiments, samples were collected and immediately preserved to quantify abundance of zooplankton found in the exchange and control tanks. Changes in abundance were used to estimate the efficacy of exchange. Samples collected from the ozone tanks were preserved for analyses following an assessment of condition.

6.8 Phytoplankton

Dr. Richard Lacouture, Academy of Natural Sciences Environmental Research Center, using the following approach, analyzed all samples collected for phytoplankton analyses. Using a sub-sample from each of the samples, the number of cells present for each species (or lowest taxonomic unit) was counted directly under a compound microscope. First, 200 individual cells were counted for each of 20 files at 500x magnification. This provided data for the number of cells for small species (e.g., microflagellates and dinoflagellates). Second, 20 fields were also examined at 312x magnification, to estimate the number of larger, rarer, forms.

To measure the effect of ozone treatment, changes in concentration (before and after treatment) in the experimental ozone treatment and control tanks were compared. For this comparison, the counts were pooled across taxa to obtain total concentrations of three major groups: dinoflagellates, microflagellates, and diatoms. Although species-level information is also available, the effects on the level of taxonomic group were compared, because the species composition will vary across replicate experiments (i.e., community composition varies in space and time). Thus, this approach allows us to treat each experimental run as a replicate measure and to test for overall effects of ozone treatment across replicates. In contrast, since the community composition will differ among experimental runs, it may not be possible to compare performance at a lower taxonomic level across replicate experiments. Furthermore, this approach (using major taxonomic groups) is similar to the analyses for zooplankton and microbial components of the study.

The species-specific counts also are being used to measure the effect(s) of exchange, whereby changes in the concentration of abundant coastal forms are compared between the control tank and a third, experimental exchange tank (Section 6.4).

Since stains were not used to determine viability, the data collected in these initial experiments measured changes in the number of cells present for each group. Although this measure can easily detect significant mortality and degradation of dinoflagellates and microflagellates, it is much less informative about diatoms, for which silica cells walls (termed "frustules") can remain intact and blur the distinction between live and dead cells. Thus, this approach provides a good coarse measure only for the first two groups and not diatoms.

6.9 Laboratory Ozone Toxicity Tests

The effects of ozone were tested on marine vertebrates and invertebrates including juvenile sheepshead minnows (*Cyprinodon variegatus*), larval topsmelt (*Atherinops affinis*),

adult mysid shrimp (*Americamysis bahia*), and adults of two amphipod species (*Leptocheirus plumulosus*, and *Rhepoxinius abronius*). All organisms except *R. abronius* were received in good condition from Aquatic Biosystems, Inc. Transport water chemistry was measured upon arrival at ENSR. The range of temperatures was 18-22 ˚C, the range of pH was 7.9-8.5 and the range of salinity was 24-32 ‰. *R. abronius* were collected in the field near Anacortes, WA, shipped overnight, and received in good condition from P. Dinnel at University of Western Washington, Anacortes. Transport water was analyzed upon arrival (11 ^{o}C, pH 6.8, 31 ‰).

On the day prior to testing, 2.5-gallon or 5-gallon glass aquaria were placed in a 21-25 ˚C water bath and filled with reconstituted waters produced by adding Forty Fathoms Crystal Sea (Marine Enterprises International, Baltimore, MD) to Milli-Q water. Salinity values of reconstituted waters ranged from 28-32 ‰. For testing of *R. abronius*, the water bath was set at 15 ˚C. The water bath was covered with a frame with a plexiglass top and plastic flaps on the sides and aquaria were left overnight for equilibration of temperature. Salinity values in aquaria on testing days ranged from 29-31 ‰.

Ozone was dispensed using a Nutech 03, Inc. (McLean, VA), Model SC-10 ozone generator. Total flow through the system was 2500 mL/minutes. Flow to each tank was controlled with a flow meter (Gilmont Instruments, Tube Number NO12-10 with glass float). Nominal flow rates for experiments where 5-gallon tanks were used (*C. variegatus, R. abronius, A. affinis*) were 40, 30, 20, and 10, which corresponded to 97.5, 63.2, 38.6, and 20 mL/minutes of ozone gas. These corresponded to ozone loading rates of 0.43, 0.28, 0.17, and 0.09 mg O_3/L/minutes. The controls received compressed, ambient air at 97.5 mL/minutes (nominal flow rate of 40). Nominal flow rates for experiments where 2.5-gallon tanks were used (*A. bahia, L. plumulosus*) were 20, 15, 10, and 5, which corresponded to 38.6, 28.3, 20, and 13.1 mL/minutes of ozone gas. These corresponded to ozone loading rates of 0.34, 0.25, 0.17, and 0.11 mg O_3/L/minutes. The controls received compressed, ambient air at 38.6 ml/minutes (nominal flow rate of 20). The smaller tanks were used to facilitate counts of organisms.

Each test included a total of five chambers, with one chamber tested per treatment. Ten organisms were placed in each chamber. Small pieces of nylon mesh (five pieces, approximately 2 in. x 2 in.) were also placed in each chamber for *R. abronius* and *L. plumulosus* as substrate. Before the initiation of ozone treatment, temperature, dissolved oxygen (DO), and pH were measured for each chamber. Total residual oxidants and ORP were measured in each chamber for up to five hours at approximately 0, 0.5, 1, 2, 3, 4, and 5 hours. Total residual oxidants were reported as total residual chlorine (TRC) as measured by a Hach Pocket Colorimeter using a DPD/KI method (APHA 1998). This procedure was equivalent to the U.S.E.P.A. methods 330.5 for wastewater and standard method 4500-Cl G for drinking water. Oxidation-reduction potential was measured by using an Orion 290A meter with a Cole-Palmer Combination ORP probe (Pt electrode, Ag/AgCl reference cell). Experiments were terminated within the five-hour period if organisms in all treatments receiving ozone were moribund. Counts of survivors were conducted at the same time as chemistry measurements were made. In exposures of *A. affinis* and *R. abronius*, DO was measured at 2 hours and 4 hours, if the test was not previously terminated.

A preliminary experiment, testing for the effects of Mud-Out® Marine Mud Remover (Northeast Technical Services Co., Olmsted Falls, OH) on TRO and ORP measurements, was conducted using two 10-gallon tanks and laboratory-produced seawater. Flows to both tanks were 97.5 mL/minutes corresponding to an ozone-loading rate of 0.43 mg O_3/L/minutes. At the initiation of ozone treatment, temperature, DO, and pH were measured in each chamber. Salinity was 32 ‰. Total residual oxidants and ORP were measured at 0.33, 0.66, 1, 2, and 3 hours.

Data were analyzed for LC50 or EC50 values using the Trimmed Spearman-Karber test. An overall LC50 or EC50 was calculated for each species using 9-10 ORP concentrations recorded across all ozone flows during the duration of the exposure.

Post-exposure recovery test. Biological effects of the exposure of organisms to ozonated water were examined using 10-day old *A. bahia*. Organisms were received in good condition from Aquatic Biosystems, Inc.. Transport water chemistry was measured upon arrival at ENSR. Water temperature was 23 ºC, pH was 7.9 and salinity was 28 ‰. Organisms were placed at 19 ºC for 2 hours to acclimate to temperature.

On the day prior to testing, 5-gallon glass aquaria were placed in a 19 ± 2 ºC water bath. Each aquarium was filled with 16 L of reconstituted seawater (Forty Fathoms Crystal Sea, Marine Enterprises International, Baltimore, MD, reconstituted in Milli-Q water). Salinity of the water was 29 ‰. The water bath was covered with a frame with a plexiglass top and plastic flaps on the sides and aquaria were left overnight for equilibration of temperature. Salinity values in aquaria on testing days were 29 ‰.

Ozone was dispensed using a Nutech 03, Inc., Model SC-10 ozone generator as described previously. Ozone flow rates were 97.5, 63.2, 38.6, and 20 mL/minute. These corresponded to ozone loading rates of 0.43, 0.28, 0.17, and 0.09 mg O_3/L/minute. The control received compressed, ambient air at 97.5 mL/minute.

Each test included five chambers with one chamber tested per treatment. Ten organisms were placed in each chamber. At the initiation of ozone treatment, temperature, DO, pH, TRO, and ORP were measured in each chamber. A Hach Pocket Colorimeter and DPD/KI reagent were used to measure TRO, which was reported as mg/L TRC. Oxidation-reduction potential was measured using an Orion 290A meter with a Cole-Palmer Combination ORP probe (Pt electrode, Ag/AgCl reference cell). Measurements of ORP and TRO were again made at approximately 75 minutes. At 90 minutes, the test was terminated and survivors were siphoned out of the tanks. They were placed in 250-ml beakers with 200 ml of clean seawater (29 ‰). Organisms were fed *Artemia franciscana* (100 µL/beaker) and were placed in a 19 ± 2 ºC water bath. After 24 hours, organisms were checked for mortality or moribund conditions. Dead organisms were removed. Organisms were fed *A. franciscana* and were placed back in a 19 ± 2 ˚C water bath. After 48 hours, organisms were again checked for mortality or movement. The experiment was then terminated.

Latent toxicity test. The effects of ozonated water on 8-day old *A. bahia* were examined immediately after ozonation, 24 hours after ozonation, and 48 hours after ozonation. *A. bahia* were received in good condition from Aquatic Biosystems, Inc. on three consecutive days.

Transport water chemistry was measured upon arrival at ENSR. Water temperature was 24 ºC, pH was 7.6, and salinity ranged from 25-28 ‰. Organisms were removed to a beaker and placed in a 20 ˚C chamber to acclimate to temperature. They remained in the chamber for 2 hours.

A 5-gallon glass aquarium was filled with approximately 16 L of reconstituted water (Forty Fathoms Crystal Sea in Milli-Q water) and placed in a 19 ± 2 ºC water bath. Salinity of the water was 28 ‰. The water bath was covered with a frame with a plexiglass top and plastic flaps on the sides and the aquarium was left to equilibrate to 19 ºC. After equilibration, ozone was dispensed as described previously. The ozone flow rate to the single aquarium was 97.5 ml/minute, corresponding to an ozone-loading rate of 0.43 mg O_3/L/minute. Total residual oxidants and ORP were measured at 1 hour and 1.5 hours. Targeted TRO and ORP values for the latent toxicity test were greater than 4.0 mg/L and greater than 700 mV. At 1 hour, TRO and ORP were 3.34 mg/L and 744 mV. At 1.5 hours, values were 5.20 mg/L and 755 mV; ozonation thus was terminated at 1.5 hours. All 16 L were removed from the aquarium into a 20-L low-density polyethylene Cubitainer (Hedwin Corporation, Laporte, IN).

Exposure concentrations were mixed using ozonated water and reconstituted seawater. Percentage mixtures were 100 (ozonated water only), 75, 50, 25, and 0 (seawater only). For each mixture, three 500-ml glass beakers containing 300 mL each were prepared. A portion of each mixture was set aside for chemistry measurements. Temperature, pH, DO, conductivity, salinity, ORP, and TRO were measured for each. *A. bahia* were added at 10 organisms/beaker. Mysids were fed *A. franciscana* at 0.2 ml/beaker at the time of test initiation. Beakers were placed in a 19 ± 2 ºC water bath and loosely covered with plexiglass. After 24 hours, counts of mortalities were conducted. Dead organisms were removed from beakers. Water samples from each set of three replicates were composited. Temperature, pH, DO, conductivity, salinity, ORP, and TRO were measured. Mysids were fed *A. franciscana* at 0.1 mL/beaker. Beakers were returned to the 19 ± 2 ºC water bath and loosely covered. After 48 hours, dead organisms were again counted and water chemistry parameters were measured. The test was then terminated.

The procedure described above was conducted for water collected immediately after ozonation into the 20-L Cubitainer (0 hours), water collected and then held 24 hours in the same 20-L Cubitainer (24 hours), and water collected and then held 48 hours in the same 20-L Cubitainer (48 hours). Each test required 2.5 L of ozonated seawater. Ozonated water was stored in the Cubitainer at 12 ºC with no headspace. Stored ozonated water was warmed to 19 ºC before mixing and adding to the 24 hours and 48 hours tests. Seawater was at 20 ± 2 ºC before mixing.

Data were analyzed for L50 values using the Trimmed Spearman-Karber test. Values were calculated as a function of % ozonated water. ORP and TRO values measured immediately after mixing. The mortality data used were those collected at 48 hours.

Tubing study. A test was conducted to determine if the tubing placed in ballast tanks to collect water samples in future experiments might affect TRO or ORP measurements. A 5-gallon glass aquarium was filled with approximately 16 L of reconstituted water (Forty Fathoms Crystal Sea in Milli-Q water) and placed in a 19 ± 2 ºC water bath. Salinity of the water was 28 ‰. The water bath was covered with a frame with a plexiglass top and plastic flaps on the sides

and the aquarium was left to equilibrate to 19 ºC. After equilibration, ORP and TRO were measured. Then ozone was dispensed as described previously. The ozone flow rate to the single aquarium was 97.5 ml/minute, corresponding to an ozone-loading rate of 0.43 mg O_3/l/minute. Oxidation-reduction potential was measured at 1 hour to determine if the value was greater than 700 mV.

A 100-foot section of 3/8 x 0.62 inch polyethylene tubing (US Plastic Corporation, Lima, OH) was flushed with 12 L of Milli-Q water followed by 12 L of seawater. Sampling began after the ORP measurement was taken at 1 hour. Three samples were taken from the upper third of the water column. Then, this section was siphoned and three samples were taken at the end of the siphon. ORP and TRO were measured in all six samples. This procedure was repeated for the middle third and bottom third of the water column. All samples were collected and tested between 1 hour and 2 hours. Ozone production was halted at 2 hours.

TRO and ORP measurements of samples from the water column were compared by calculating the statistic called the Students t-test to TRO and ORP of samples collected after siphoning for the upper, middle and lower third of the aquarium. Differences were considered significant at $p < 0.05$.

6.10 Ozone Experiment

Sampling Locations and Frequency. Given that the maximum time available for ballast tank ozonation during a typical voyage from Port Angeles to Valdez, AK is 3.5 days, it has been estimated that each ballast tank may only be ozonated for a maximum of 5 hours. This estimate assumes: 1) all the ballast tanks are full; 2) all tanks are going to be discharged into coastal waters; and thus 3) all ballast water requires treatment. Longer ozonation periods may also be possible to achieve, so testing entailed both a single 5-hour exposure to provide a conservative evaluation of ozonation effectiveness at this minimum exposure time (experiment 1 in September 2001), as well as two 10-hour exposures to evaluate effectiveness during longer exposure times (experiments 2 and 3 in November 2001).

Sampling locations and frequency depended on the type of data being collected and varied somewhat between the three experiments. For simplicity in this initial study, reliance was placed primarily upon grab samples and vertical net tows collected from several vertical access points (manways or Butterworth® openings) in the treatment and control tanks. All samples for water chemistry and microbiota were collected at the beginning and end of the ozonation period, as well as at 2.5-hour increments to evaluate effectiveness at intermediate times. Larger organisms (e.g., plankton tows) were collected from two different vertical Butterworth® hatches in each tank at the 0 hours and 5 hours time points, as well as at the 10 hours time points during the 10-hour experiments (Figure 6.1.1). Caged organisms were also placed at four depths below two or three of the vertical access points. Finally, composite samples were collected from several discrete Niskin samples for use in WET testing.

*Chemistry and microbiology sampling***.** Samples were collected from each tank using Niskin water bottles vertically deployed through two Butterworth® openings (Figure 6.1.1). Samples were collected from three different depths in the vertical side tanks: 10 feet below the

surface of the water (depth 1); 30 feet below the surface (depth 2); and 50 feet below the surface (depth 3). Total water column depth varied between tanks and experiments, but averaged about 65 feet.

Plankton sampling. Plankton tows were obtained from two vertical openings over each ballast tank (except for the two 10-hour experiments, where the control tank was sampled through only one access port). Triplicate tows were taken immediately prior to ozonation, after five hours of ozonation, and also, during the 10-hour experiments, after ten hours of ozonation.

Caged organism sampling. The abundance and taxonomic composition of the ballast water organisms collected during tank fill cannot be predicted or repeated, especially for large mobile organisms. Therefore, caged organisms were introduced to the treatment and control tanks during the ozone experiments to provide known and repeatable biotic assemblages against which ozonation effectiveness could be tested for this group. Caged organisms included mysids, amphipods, shore crabs, and sheepshead. Each was suspended from manhole access ports prior to the start of ozonation for retrieval after completion of ozonation.

Overall sampling frequency. Sampling frequency depended on sample type (Tables 6.10.1 and 6.10.2). More sampling effort was concentrated at the beginning and end of the study, but some chemical and biological data were collected (on a limited basis) mid-way through the experiment. Ozone chemistry and microbes (bacteria counts) were sampled frequently to track changes in ozone chemistry (and its residuals), and biological responses obtained from the same samples. These samples were collected from the deck via Niskin samplers (Table 6.10.1). Samples from plankton tows were collected at the beginning and end (0 hours and 5 hours) of the 5-hour ozone experiment and at the beginning, midpoint and end (0 hours, 5 hours, 10 hours) of the 10-hour ozone experiments (Table 6.10.2). Caged organisms were suspended from Butterworth® openings in the tanks just prior to ozonation, and collected as soon as possible after treatment was completed for the counting of living/moribund/dead organisms. Water samples for WET testing and chemistry splits were also collected using Niskin grabs from both ballast tanks immediately after completion of ozonation.

As shown in Tables 6.10.1 and 6.10.2, sampling times for the control and treatment tanks was staggered to facilitate efficient use of personnel. This eliminated the logistically difficult task of sampling both the control and treatment tanks simultaneously, and ensured that time-sensitive ozone chemistry measurements (e.g., ozone, bromine, ORP) could be taken within minutes of collection. While this offset the experiment initiation in each tank to a minor degree, this should not have substantially impacted the results because the control did not use any kind of aeration in this experiment. Specific personnel assignments, volumes required, and materials needed for Niskin sampling are included in Appendix A.

Table 6.10.1. Description and collection schedule for samples collected via Niskin grabs. Numbers at individual time points denote samples per individual Niskin grab. Totals represent all samples collected from each of two vertical access ports per tank, and collected from each of three different depths.

		Basic Water Quality Parameters							Ozone and Residuals					Biology			Toxicity
Analyte		Salinity	Temp	DO	pH	Nitrite	Nitrate	React. Phos.	Ozone	Bromine	ORP	Bromate	Bromoform	Bacteria	Phytoplankton	Zooplankton	
Analytical Method		Conductivity Meter	pH meter	DO meter	pH meter	Std. Colorimetric	Std. Colorimetric	Std. Colorimetric	Accuvac	Accuvac	probe*	Ion chrom.	GC	direct count	direct count	direct count	WET test
Location Analyzed		on board	on board	on board	on board	lab	lab	lab	on board	on board	on board	lab	lab	lab	lab	on board	lab
Hour	Tank	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples	No. of samples
-1	control	3	3	3	3	3	3	3	6	6	3	3	6	3	3	3	
0	treatment	3	3	3	3	3	3	3	6	6	3	3	6	3	3	3	
1.5	control	3	3	3	3				6	6	3	3	6	3	3	3	
2.5	treatment	3	3	3	3				6	6	3	3	6	3	3	3	
4	control	3	3	3	3				6	6	3	3	6	3	3	3	
5	treatment	3	3	3	3				6	6	3	3	6	3	3	3	
6.5	control	3	3	3	3				6	6	3	3	6	3	3	3	
7.5	treatment	3	3	3	3				6	6	3	3	6	3	3	3	
9	control	3	3	3	3				6	6	3	3	6	3	3	3	
10	treatment	3	3	3	3				6	6	3	3	6	3	3	3	
10.5	treatment																2

Table 6.10.2. Description and schedule for samples to be collected via vertical plankton net tows. Sample numbers at each time period indicate total number of samples to be collected.

Tank		Zooplankton Sps. Composition and Abudnance
Method:		Direct microscope count
Analysis:		Live vs. dead, and preserved?
Location:		On Board, UW
Time:		
-1 Hr	Treatment	2 columns * 3 replicates = 6
0 Hr (begin ozonation)		
1Hr	Control	6
2.5 Hr	Treatment	6
3.5 Hr	Control	6
5 Hr (stop ozone)	Treatment	6
6Hr	Control	6
TOTAL SAMPLES =		**24**

6.11 Ozone Treatment Control

An experimental control for ozonation could consist either of bubbling ambient air through ozone diffusers at approximately the same rate as ozone-containing gas, or no treatment of any kind. Aeration would mimic the physical disturbance and water column mixing of the ozone treatment, but it may be possible that aeration could also negatively impact some planktonic organisms. Therefore, for this preliminary study, we selected a no-treatment control. However, critically important to the success of this design was that both the ozonated and control tanks were filled at the same time and with the same water mass.

6.12 Caged Organism Studies

In situ caged organism exposures were employed to evaluate the efficacy of ozone ballast water treatment across a range of aquatic organisms. Prior to initiation of ozone treatment, test organisms were placed in cages and suspended via a tether line in both the ozone-treated and control tanks. Organisms remained in the ballast water tanks throughout the 5- or 10-hour ozone exposures after which they were evaluated for survival and morbidity. A variety of vertebrate and invertebrate aquatic organisms were evaluated including: mysid shrimp (*A. bahia*), sheepshead minnows (*C. variegatus*), shore crab (*Hemigrapsus nudus*), and amphipod (*R. abronius)*. These organisms were chosen based on their known sensitivity or hardiness (shore crabs) to a variety of aquatic toxicants and their use as "standard" laboratory test organisms. Organisms were obtained from a commercial supplier in Fort Collins, CO (mysids and sheepshead) or field collected from areas near Anacortes, WA (shore crabs and amphipods). All organisms were acclimated and maintained under either static or flowing seawater conditions at Western Washington University's Shannon Point Marine Laboratory, Anacortes, WA. Prior to testing, organisms were placed in individual exposure chambers and transported to the *S/T Tonsina* in ice chests containing aerated seawater.

Groups of caged organisms were placed into the control and treatment tanks. Each exposure group consisted of a plastic bucket containing sand (Figure 6.12.1) connected to a tether rope by which it could be lowered through the access hatches to the bottom of the ballast water tank. Buckets were used for deployment of amphipod exposure chambers, with chambers for the other three species being suspended from the tether rope at 10, 30 and 50 feet above the bottom (Figure 6.12.2).

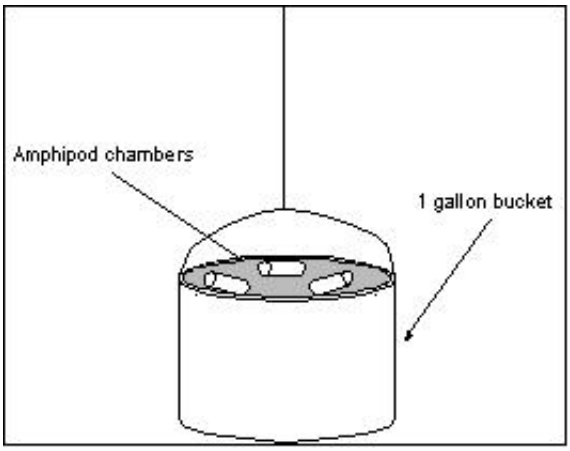


Figure 6.12.1. Close-up of the bucket with replicate amphipod chambers.

For the amphipods, three *in situ* chambers were put into the top half of each bucket (sand in the bottom half acted as anchors) (Figure 6.12.1). Amphipod chambers were modeled after that described by Tucker and Burton (1999), and contained 10 amphipods each (30/bucket). Amphipod chambers were constructed of 5-cm diameter clear plastic tubes approximately 12-cm long capped at each end with polypropylene caps. Each chamber contained two rectangular 3 x 5-cm openings covered with 1-mm polypropylene-woven screen and held in place using silicon glue. All exposure chambers were soaked in both freshwater and seawater for 24 hours each to assure that chamber construction materials or the silicon glue did not impart any toxicity. Amphipod exposure chambers were held in the plastic buckets by means of a coarse mesh polyethylene net placed around each bucket.

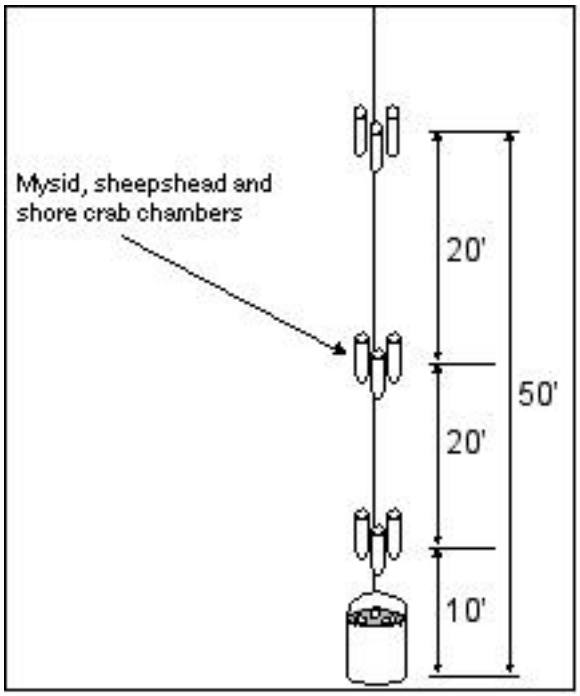


Figure 6.12.2. Drawing of one complete set of exposure chambers.

Exposure chambers for each of the other three test species (i.e., mysid shrimp, sheepshead minnow, and shore crab) were attached to the tether rope at intervals of 10, 30, and 50 feet above the bucket (Figure 6.12.2).

For mysids and sheepshead, ten individual organisms (of a single species) were placed into the clear-plastic exposure chambers, constructed as above and containing two rectangular windows (3 x 5 cm) covered with 750-μm mesh (for mysids) or 1-mm mesh (for sheepshead), one on either side of the chamber, and each chamber was capped with polypropylene caps. For shore crabs, ten individual organisms were placed into commercially available plastic crab bait buckets (11 cm high x 9 cm diameter) drilled with numerous 8 mm holes. Groups of three chambers (one for each species) were placed in coarse-mesh polyethylene nets and attached to the tether rope via clamps.

At the completion of the 5- or 10-hours ozone treatment periods, cages were removed and the number of surviving organisms recorded immediately. Additionally, numbers of animals appearing moribund (or failing to rebury in sand for the amphipods) were also recorded. Results are reported as percent survivorship and percent moribund for each taxon.

7 RESULTS AND DISCUSSION

7.1 Ozone Delivery

Table 7.1.1 summarizes the water volume capacity of both sections of the ozone treatment tank (No. 3 port ballast tank) and number of ozone diffusers in each section, as well as the calculated ozone loading rate in each section for each of the three experiments. Note that the "vertical/sample portion 3P" row shows the information pertaining to the vertical wing tank that is the portion from which samples for these experiments were taken. The ozone loading rate in this wing tank increased by 22 % between experiments 1 and 2, and then by 87.5 % between experiments 2 and 3. This increase in ozone loading is generally reflected in the biological and chemical data presented below. The low number of diffusers in this wing tank is notable, given the variability of effectiveness within the tank that the biological data reveal.

7.2 General Chemical Characteristics

Several general water quality parameters were recorded during the three experiments. These water quality parameters were dissolved oxygen (Table 7.2.1), pH (Table 7.2.2), salinity (Table 7.2.3) temperature, (Table 7.2.4), dissolved organic carbon (Table 7.2.5), phosphate ion (Table 7.2.6), silica (Table 7.2.7), nitrate ion (Table 7.2.8), nitrite ion (Table 7.2.9) and ammonia (Table 7.2.10).

Dissolved oxygen is generally considered an important parameter in water quality to sustain aerobic life. In general, highly oxygenated water is considered "healthy" for biota. Because oxygen is the stable product of ozone, it was measured to determine whether its concentration would increase with ozonation.

Another generally important water quality parameter is pH. This provides a measure of the acid/base equilibrium in water. In the case of ozonation, it also is important in determining the distribution of the reaction by-product of ozone and bromide ion, bromine.

Salinity is the measure of the total amount of "salts" in water. The measure of salinity provides an estimate of the amount of oceanic water in the ballast water. Of interest in the ozonation of seawater are the reactions of ozone with bromide ion. It was thought that no change in salinity would occur during ozonation.

The organic fraction of the water (DOC) is of interest because: 1) it provides an indication of the quality of the water being used for ballast purposes (the lower the DOC the higher the water quality), and, 2) the reaction of bromine with DOC leads to the formation of bromoform, which is one of the ozone reaction by-products of potential interest.

The nutrient data are indicative of general ballast water quality, and the potential of this water to support the growth of phytoplankton and other microorganisms.

Table 7.1.1 Ozone production, distribution and loading in the treatment tank.

				diffuser density	O3 production			O3 distributition			O3 loading rate		
Ballast Tank	volume (BBLS)	volume (L)	No. of O3 diffusers	(BBLS per diffuser)	Exp 1 gram/hr	Exp 2 gram/hr	Exp 3 gram/hr	Exp 1 %	Exp 2 %	Exp 3 %	Exp 1 (mg/l/hr)	Exp 2 (mg/l/hr)	Exp 3 (mg/l/hr)
3P	19,608	3,117,084	72	272	1460	1760	1660				0.47	0.56	0.53
horizontal portion 3P	11,802	1,876,164	56	211				50%	40%	0%	0.39	0.38	0.00
vertical/sample portion	3P,723	1,227,725	16	483				50%	60%	100%	0.59	0.72	1.35
average Tonsina tank	*17,925*	2,849,537						*100%*	*100%*	*100%*	*0.51*	*0.62*	*0.58*

Table 7.2.1. Summary of the results of dissolved oxygen (DO) in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		DO (mg/L as O_2)		
A10- Treatment	T-0.0	Not Sampled	9.0	6.6
	T-2.5	Not Sampled	10.3	13.8
	T-5.0	Not Sampled	14.3	17.5
	T-7.5	Not Sampled	Not Sampled[1]	19.1
	T-10.0	Not Sampled	21.8	20.2
A30- Treatment	T-0.0	Not Sampled	9.2	6.8
	T-2.5	Not Sampled	10.5	12.7
	T-5.0	Not Sampled	14.5	19.2
	T-7.5	Not Sampled	Not Sampled	19.2
	T-10.0	Not Sampled	21.5	19.2
A50- Treatment	T-0.0	Not Sampled	8	7.8
	T-2.5	Not Sampled	7.9	8.3
	T-5.0	Not Sampled	15.3	13.1
	T-7.5	Not Sampled	Not Sampled	16.5
	T-10.0	Not Sampled	14.2	18.1
B10- Treatment	T-0.0	Not Sampled	8.9	6.1
	T-2.5	Not Sampled	8.4	7.8
	T-5.0	Not Sampled	11.9	15
	T-7.5	Not Sampled	Not Sampled	17.4
	T-10.0	Not Sampled	14.9	19.6
B30- Treatment	T-0.0	Not Sampled	9.3	6.3
	T-2.5	Not Sampled	8.6	8.6
	T-5.0	Not Sampled	11.5	16.8
	T-7.5	Not Sampled	Not Sampled	17
	T-10.0	Not Sampled	18.8	18.8
B50- Treatment	T-0.0	Not Sampled	9.3	6.9
	T-2.5	Not Sampled	7.8	8.8
	T-5.0	Not Sampled	10.6	15.2
	T-7.5	Not Sampled	Not Sampled	16.3
	T-10.0	Not Sampled	19	18.2
C10-Control	T-0.0	Not Sampled	5.9	6.9
	T-2.5	Not Sampled	6.5	7.1
	T-5.0	Not Sampled	6.6	7.4
	T-7.5	Not Sampled	Not Sampled	8
	T-10.0	Not Sampled	8	6.4
C30-Control	T-0.0	Not Sampled	5.8	6.2
	T-2.5	Not Sampled	6.5	6.2
	T-5.0	Not Sampled	6.1	7.9
	T-7.5	Not Sampled	Not Sampled	8.2
	T-10.0	Not Sampled	7.7	6.4

Table 7.2.1. Summary of the results of dissolved oxygen (DO) in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
C50-Control	T-0.0	Not Sampled	5.7	5.9
	T-2.5	Not Sampled	6.3	6.9
	T-5.0	Not Sampled	6.4	8.2
	T-7.5	Not Sampled	Not Sampled	7.3
	T-10.0	Not Sampled	6.9	7.4
D10-Control	T-0.0	Not Sampled	Not Sampled	Not Sampled
	T-2.5	Not Sampled	Not Sampled	Not Sampled
	T-5.0	Not Sampled	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	Not Sampled	Not Sampled	Not Sampled
	T-2.5	Not Sampled	Not Sampled	Not Sampled
	T-5.0	Not Sampled	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D50-Control	T-0.0	Not Sampled	Not Sampled	Not Sampled
	T-2.5	Not Sampled	Not Sampled	Not Sampled
	T-5.0	Not Sampled	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
[1] During the second experiment, the fourth time interval there was apparently a problem with the DO meter and all of the samples were lost.				

For the two experiments where DO was measured, Experiment 2 and 3, a steady increase was observed in the ballast tanks that were ozonated (Table 7.2.1). The T1 samples for Experiment 2 averaged 8.95 mg/L of O_2 whereas the T1 samples for experiment three averaged 6.75. In both experiments the O_2 concentration increased by at least two-fold during the ozonation period and showed a steady increase with increased ozonation time. This is consistent with ozone decomposition into O_2. The control tank that was sampled showed no consistent pattern of O_2 change.

This increase in O_2 concentration would have a positive effect on water quality for disposal, or dumping in the receiving port of call. An interesting question would be to determine how long the elevated concentration of O_2 remained in the ballast water in closed tanks. It is possible that this dramatic increase in O_2 concentration might have an adverse affect on some organisms entrained in the water at the time of filling. In particular any anaerobic bacteria or organisms that have a low threshold for elevated O_2 concentrations would presumably not survive in this environment for very long.

Table 7.2.2. Summary of the results of pH measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		pH		
A10- Treatment	T-0.0	7.5 – 8.0	7.4	7.54
	T-2.5	7.0	7.5	7.7
	T-5.0	7.0	7.5	7.7
	T-7.5	Not Sampled	7.8	7.8
	T-10.0	Not Sampled	7.9	7.8
A30- Treatment	T-0.0	7.0 – 7.5	7.42	7.55
	T-2.5	7.0 – 7.5	7.51	7.86
	T-5.0	7.0 – 7.5	7.55	7.72
	T-7.5	Not Sampled	7.83	7.77
	T-10.0	Not Sampled	7.95	7.82
A50- Treatment	T-0.0	7.0 – 7.5	7.43	7.54
	T-2.5	7.0	7.5	7.6
	T-5.0	7.0 – 7.5	7.54	7.83
	T-7.5	Not Sampled	7.7	7.7
	T-10.0	Not Sampled	7.9	7.89
B10- Treatment	T-0.0	7.5 – 8.0	7.44	7.53
	T-2.5	7.0	7.5	7.6
	T-5.0	7.0 – 7.5	7.49	7.7
	T-7.5	Not Sampled	7.7	7.7
	T-10.0	Not Sampled	7.89	7.79
B30- Treatment	T-0.0	7.0 – 7.5	7.39	7.54
	T-2.5	7.5	7.5	7.6
	T-5.0	7.0 – 7.5	7.49	7.72
	T-7.5	Not Sampled	7.8	7.8
	T-10.0	Not Sampled	7.91	7.83
B50- Treatment	T-0.0	7.0 – 7.5	7.45	7.54
	T-2.5	7.0	7.5	8.0
	T-5.0	7.0	7.5	7.7
	T-7.5	Not Sampled	7.7	7.8
	T-10.0	Not Sampled	7.9	7.8
C10-Control	T-0.0	7.5	7.32	7.97
	T-2.5	7.0 – 7.5	7.46	7.78
	T-5.0	7.5	7.47	7.61
	T-7.5	Not Sampled	7.7	7.64
	T-10.0	Not Sampled	7.74	7.01
C30-Control	T-0.0	7.0 – 7.5	7.46	7.75
	T-2.5	7.0	7.5	7.9
	T-5.0	7.0 – 7.5	7.44	7.63
	T-7.5	Not Sampled	7.7	7.6
	T-10.0	Not Sampled	7.71	7.7

Table 7.2.2. Summary of the results of pH measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		pH		
C50-Control	T-0.0	7.0 – 7.5	7.45	7.76
	T-2.5	7.0	7.5	7.7
	T-5.0	7.0 – 7.5	7.46	7.84
	T-7.5	Not Sampled	7.7	7.7
	T-10.0	Not Sampled	7.73	7.67
D10-Control	T-0.0	7.0 – 7.5	Not Sampled	Not Sampled
	T-2.5	7.0 – 7.5	Not Sampled	Not Sampled
	T-5.0	7.0 – 7.5	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	7.0 – 7.5	Not Sampled	Not Sampled
	T-2.5	7.0	Not Sampled	Not Sampled
	T-5.0	7.0 – 7.5	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D50-Control	T-0.0	7.5	Not Sampled	Not Sampled
	T-2.5	7.5	Not Sampled	Not Sampled
	T-5.0	7.0 – 7.5	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled

The pH of the ballast water in the ozonated tanks increased slightly by about 0.3 – 0.4 units during the course of the 10-hour experiments (Experiment 2 and 3) but did not increase in the 5-hour experiment (Experiment 1; Table 7.2.2). pH in the control tanks showed no increase in any of the 5 or 10-hours experiments. These minor fluctuations will probably not have any positive or adverse affects on the chemistry of the ozone, or on the organisms in the ballast tanks.

Table 7.2.3. Summary of the results of salinity measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Salinity (‰)		
A10- Treatment	T-0.0	33.7	35.6	34.1
	T-2.5	33.5	35.3	34.1
	T-5.0	33.6	35.1	33.9
	T-7.5	Not Sampled	35.3	34.0
	T-10.0	Not Sampled	35.2	33.9
A30- Treatment	T-0.0	33.5	35.8	34.1
	T-2.5	33.6	35.3	34.2
	T-5.0	33.5	35.1	34.1
	T-7.5	Not Sampled	35.1	34.2
	T-10.0	Not Sampled	35.1	34.3
A50- Treatment	T-0.0	33.5	35.6	34.1
	T-2.5	33.6	35.3	34.4
	T-5.0	33.4	35.1	34.2
	T-7.5	Not Sampled	35.1	34.3
	T-10.0	Not Sampled	35.1	34.2
B10- Treatment	T-0.0	33.6	35.9	34.2
	T-2.5	33.3	35.4	34.2
	T-5.0	33.5	35.1	34.1
	T-7.5	Not Sampled	35.0	34.1
	T-10.0	Not Sampled	35.1	34.2
B30- Treatment	T-0.0	33.7	35.7	33.9
	T-2.5	33.0	35.4	34.2
	T-5.0	33.5	35.1	34.2
	T-7.5	Not Sampled	35.1	34.2
	T-10.0	Not Sampled	35.1	34.1
B50- Treatment	T-0.0	33.6	35.7	34.3
	T-2.5	33.7	35.3	34.4
	T-5.0	33.4	35.2	34.3
	T-7.5	Not Sampled	35.1	34.2
	T-10.0	Not Sampled	35.1	34.1
C10-Control	T-0.0	33.2	35.4	34.0
	T-2.5	33.5	35.4	34.0
	T-5.0	33.5	35.2	34.0
	T-7.5	Not Sampled	35.0	34.1
	T-10.0	Not Sampled	35.2	33.9
C30-Control	T-0.0	33.5	35.5	34.2
	T-2.5	33.6	35.1	34.3
	T-5.0	33.5	35.3	34.2
	T-7.5	Not Sampled	35.3	34.3
	T-10.0	Not Sampled	35.4	34.1

Table 7.2.3. Summary of the results of salinity measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Salinity (‰)		
C50-Control	T-0.0	33.5	35.2	34.2
	T-2.5	33.6	35.4	34.3
	T-5.0	33.2	35.2	34.2
	T-7.5	Not Sampled	35.4	34.3
	T-10.0	Not Sampled	35.4	34.1
D10-Control	T-0.0	33.5	Not Sampled	Not Sampled
	T-2.5	33.5	Not Sampled	Not Sampled
	T-5.0	33.6	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	33.6	Not Sampled	Not Sampled
	T-2.5	33.6	Not Sampled	Not Sampled
	T-5.0	33.5	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D50-Control	T-0.0	33.4	Not Sampled	Not Sampled
	T-2.5	33.5	Not Sampled	Not Sampled
	T-5.0	33.4	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled

Salinity was not affected by the addition of ozone to the ballast water and there was no difference between the treated and untreated (control) tanks in any of the experiments (Table 7.2.3). This is consistent with the decomposition of ozone in saline waters.

Table 7.2.4. Summary of the result of temperature measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Temperature (^{o}C)		
A10- Treatment	T-0.0	15.1	10.3	10.7
	T-2.5	13.8	11.8	10.0
	T-5.0	14.0	11.1	10.6
	T-7.5	Not Sampled	11.2	10.3
	T-10.0	Not Sampled	10.6	9.9
A30- Treatment	T-0.0	14.8	11.1	10.8
	T-2.5	13.5	11.3	11.4
	T-5.0	13.6	11.2	10.3
	T-7.5	Not Sampled	10.5	10.3
	T-10.0	Not Sampled	10.4	10.2
A50- Treatment	T-0.0	14.2	11.9	10.3
	T-2.5	13.3	10.3	10.3

Table 7.2.4. Summary of the result of temperature measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Temperature (^{o}C)		
	T-5.0	12.7	10.2	10.4
	T-7.5	Not Sampled	9.4	10.1
	T-10.0	Not Sampled	9.4	10.1
B10- Treatment	T-0.0	14.1	12.0	10.7
	T-2.5	14.1	11.9	9.9
	T-5.0	13.6	11.0	10.1
	T-7.5	Not Sampled	10.6	9.7
	T-10.0	Not Sampled	10.0	9.7
B30- Treatment	T-0.0	15.5	9.8	10.6
	T-2.5	13.2	11.6	10.0
	T-5.0	13.7	11.8	10.1
	T-7.5	Not Sampled	10.4	10.1
	T-10.0	Not Sampled	10.2	9.5
B50- Treatment	T-0.0	15.1	10.2	10.1
	T-2.5	14.1	10.4	10.8
	T-5.0	12.7	11.0	10.7
	T-7.5	Not Sampled	11.1	11.3
	T-10.0	Not Sampled	9.6	10.2
C10-Control	T-0.0	14.4	10.9	10.0
	T-2.5	13.7	10.7	10.0
	T-5.0	13.4	9.9	10.1
	T-7.5	Not Sampled	9.2	9.9
	T-10.0	Not Sampled	9.6	9.6
C30-Control	T-0.0	14.7	10.5	9.9
	T-2.5	13.9	10.6	9.9
	T-5.0	13.4	9.7	10.3
	T-7.5	Not Sampled	9.6	11.4
	T-10.0	Not Sampled	9.5	10.7
C50-Control	T-0.0	14.0	10.6	10.7
	T-2.5	14.6	10.7	10.1
	T-5.0	12.8	10.3	11.1
	T-7.5	Not Sampled	9.8	10.4
	T-10.0	Not Sampled	9.9	10.3
D10-Control	T-0.0	15.7	Not Sampled	Not Sampled
	T-2.5	13.8	Not Sampled	Not Sampled
	T-5.0	13.3	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	14.8	Not Sampled	Not Sampled
	T-2.5	13.9	Not Sampled	Not Sampled
	T-5.0	12.8	Not Sampled	Not Sampled

Table 7.2.4. Summary of the result of temperature measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Temperature (^{o}C)		
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D50-Control	T-0.0	13.6	Not Sampled	Not Sampled
	T-2.5	14.5	Not Sampled	Not Sampled
	T-5.0	12.8	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled

The temperature of the ballast water for Experiment 1 was consistently warmer than for Experiments 2 and 3 (Table 7.2.4). This presumably reflects the time of year that the experiments were conducted. During the 5-hour experiment, Experiment 1, the temperature appeared to decrease approximately one degree. During the ten-hour experiments, Experiments 2 and 3, there was no clear trend or change in temperature throughout the study. The minor variations in temperature suggest that this will have neither a positive nor a negative effect on the treatment. However, the lower temperatures may account for lower bromoform formation in Experiments 2 and 3.

Table 7.2.5 summarizes the dissolved organic carbon concentration in the treated and control tanks of the three experiments. These results suggest that the DOC concentration in the ballast water was similar for all of the experiments. Similarly, no trends were apparent as a function of time or ozone treatment in any of the inorganic nutrients (Tables 7.2.6 – 7.2.10).

Table 7.2.5. Summary of the result of dissolved organic carbon (DOC) measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		DOC (mg C /L)		
A10 - Treatment	T-0.0	0.95	0.79	1.11
A30- Treatment	T-0.0	0.93	0.85	1.02
A50- Treatment	T-0.0	0.92	0.75	0.98
B10- Treatment	T-0.0	1.01	0.91	1.02
B30- Treatment	T-0.0	0.95	0.92	1.02
B50- Treatment	T-0.0	1.00	0.82	0.84
C10- Control	T-0.0	Not Sampled	0.81	1.08
C30- Control	T-0.0	0.93	0.72	1.18
C50- Control	T-0.0	0.94	0.74	0.81
D10- Control	T-0.0	0.95	Not Sampled	Not Sampled
D30- Control	T-0.0	0.92	Not Sampled	Not Sampled
D50- Control	T-0.0	1.06	Not Sampled	Not Sampled

Table 7.2.6. Summary of the result of ortho-phosphate ion (PO_4^{3-}) measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).

Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		PO_4^{3-} (mg/L)		
A10 - Treatment	T-0.0	0.0635	0.0762	0.0762
A30- Treatment	T-0.0	0.0634	0.0759	0.0748
A50- Treatment	T-0.0	0.0638	0.0758	0.0751
B10- Treatment	T-0.0	0.0633	0.0760	0.0743
B30- Treatment	T-0.0	0.0635	0.0756	0.0746
B50- Treatment	T-0.0	0.0628	0.0763	0.0744
C10- Control	T-0.0	Not Sampled	0.0750	0.0748
C30- Control	T-0.0	0.0638	0.0753	0.0745
C50- Control	T-0.0	0.0639	0.0752	0.0745
D10- Control	T-0.0	0.0623	Not Sampled	Not Sampled
D30- Control	T-0.0	0.0636	Not Sampled	Not Sampled
D50- Control	T-0.0	0.0624	Not Sampled	Not Sampled

Table 7.2.7. Summary of the result of silica, $Si(OH)_4$, measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).

Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		$Si(OH)_4$ (mg/L)		
A10 - Treatment	T-0.0	1.333	1.519	1.508
A30- Treatment	T-0.0	1.330	1.507	1.504
A50- Treatment	T-0.0	1.322	1.494	1.505
B10- Treatment	T-0.0	1.331	1.512	1.516
B30- Treatment	T-0.0	1.325	1.498	1.489
B50- Treatment	T-0.0	1.326	1.497	1.485
C10- Control	T-0.0	Not Sampled	1.510	1.513
C30- Control	T-0.0	1.349	1.506	1.487
C50- Control	T-0.0	1.344	1.497	1.483
D10- Control	T-0.0	1.357	Not Sampled	Not Sampled
D30- Control	T-0.0	1.342	Not Sampled	Not Sampled
D50- Control	T-0.0	1.353	Not Sampled	Not Sampled

Table 7.2.8. **Summary of the result of nitrate ion (NO_3^-) measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).**

Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		NO_3^- (mg/L)		
A10 - Treatment	T-0.0	0.287	0.399	0.399
A30- Treatment	T-0.0	0.290	0.397	0.401
A50- Treatment	T-0.0	0.290	0.399	0.403
B10- Treatment	T-0.0	0.286	0.398	0.394
B30- Treatment	T-0.0	0.287	0.399	0.399
B50- Treatment	T-0.0	0.289	0.399	0.410
C10- Control	T-0.0	Not Sampled	0.399	0.395
C30- Control	T-0.0	0.294	0.399	0.412
C50- Control	T-0.0	0.294	0.399	0.402
D10- Control	T-0.0	0.289	Not Sampled	Not Sampled
D30- Control	T-0.0	0.291	Not Sampled	Not Sampled
D50- Control	T-0.0	0.291	Not Sampled	Not Sampled

Table 7.2.9. **Summary of the result of nitrite ion (NO_2^-) measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).**

Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		NO_2^- (mg/L)		
A10 - Treatment	T-0.0	0.004	0.005	0.006
A30- Treatment	T-0.0	0.004	0.005	0.005
A50- Treatment	T-0.0	0.004	0.005	0.005
B10- Treatment	T-0.0	0.004	0.006	0.006
B30- Treatment	T-0.0	0.004	0.005	0.005
B50- Treatment	T-0.0	0.004	0.005	0.005
C10- Control	T-0.0	Not Sampled	0.005	0.004
C30- Control	T-0.0	0.004	0.005	0.006
C50- Control	T-0.0	0.004	0.005	0.005
D10- Control	T-0.0	0.004	Not Sampled	Not Sampled
D30- Control	T-0.0	0.004	Not Sampled	Not Sampled
D50- Control	T-0.0	0.004	Not Sampled	Not Sampled

Table 7.2.10. Summary of the result of ammonium ion (NH_3) measurements in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		NH_4 (mg/L)		
A10 - Treatment	T-0.0	0.032	0.076	0.076
A30- Treatment	T-0.0	0.029	0.076	0.075
A50- Treatment	T-0.0	0.031	0.076	0.075
B10- Treatment	T-0.0	0.031	0.076	0.074
B30- Treatment	T-0.0	0.029	0.076	0.075
B50- Treatment	T-0.0	0.028	0.076	0.074
C10- Control	T-0.0	Not Sampled	0.075	0.075
C30- Control	T-0.0	0.028	0.075	0.075
C50- Control	T-0.0	0.028	0.075	0.075
D10- Control	T-0.0	0.027	Not Sampled	Not Sampled
D30- Control	T-0.0	0.026	Not Sampled	Not Sampled
D50- Control	T-0.0	0.027	Not Sampled	Not Sampled

7.3 Ozone/Oxidant Chemistry

7.3.1 Ozone and Bromine.

At every sampling point and time both ozone (Table 7.3.1) and bromine, hypobromous acid/hypobromite ion, (Table 7.3.2) were determined in duplicate from the same Niskin sample.

Table 7.3.1. Ozone concentration in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Ozone (mg/L)		
A10- Treatment	T-0.0	0.03, -0.08[1]	0.30, 0.32	0.01, -0.09
	T-2.5	0.56, 0.43	0.19, 0.19	-0.04, -0.01
	T-5.0	0.23, 0.26	-0.06, -0.08	0.03, 0.06
	T-7.5	Not Sampled	Not Sampled	0.09, 0.01
	T-10.0	Not Sampled	0.07, 0.25	0.07, 0.06
A30- Treatment	T-0.0	0.00, 0.00	0.39, 0.33	-0.16,0.12
	T-2.5	0.08, -0.01	0.33, 0.18	-0.05, -0.01
	T-5.0	0.20, 0.03	-0.05, -0.06	0.06, 0.04
	T-7.5	Not Sampled	Not Sampled	-0.03, -0.07
	T-10.0	Not Sampled	0.25, 0.06	0.48, 0.10
A50- Treatment	T-0.0	-0.11, -0.02	0.57, 0.32	-0.09
	T-2.5	0.15, 0.14	0.48, 0.07	-0.03, -0.01
	T-5.0	0.24, 0.26	0.49, -0.06	-0.03, -0.32
	T-7.5	Not Sampled	0.49	0.04, -0.07

Table 7.3.1. Ozone concentration in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Ozone (mg/L)		
	T-10.0	Not Sampled	0.38, 0.32	0.25, -0.04
B10- Treatment	T-0.0	0.05, 0.02	0.46, 0.31	-0.02, -0.05
	T-2.5	0.00, -0.03	0.05, 0.11	-0.08, -0.02
	T-5.0	0.10, 0.01	0.12, 0.03	0.03, -0.11
	T-7.5	Not Sampled	-0.09	-0.02, -0.02
	T-10.0	Not Sampled	0.01, 0.04	-0.02,-0.03
B30- Treatment	T-0.0	0.05, 0.02	0.30, 0.37	0.08, 0.10
	T-2.5	0.03, 0.01	0.22, 0.09	-0.06, -0.01
	T-5.0	0.02, 0.00	0.12, 0.08	-0.04, -0.18
	T-7.5	Not Sampled	-0.03	-0.11, -0.14
	T-10.0	Not Sampled	0.27, 0.01	-0.10, -0.16
B50- Treatment	T-0.0	0.01, 0.03	0.30, 0.37	-0.07, 0.01
	T-2.5	0.01, 0.00	0.35, 0.04	-0.01, -0.10
	T-5.0	0.09, 0.17	-0.04, 0.94	0.14, -0.01
	T-7.5	Not Sampled	0.04	0.00, -0.07
	T-10.0	Not Sampled	0.05, 0.01	-0.05, 0.17
C10-Control	T-0.0	-0.04, 0.03	0.50, 0.43	-0.02, -0.21
	T-2.5	0.00, 0.00	0.19, -0.28	0.00, -0.03
	T-5.0	0.24, 0.00	0.78, 0.02	0.18, -0.28
	T-7.5	Not Sampled	-0.02	-0.11, -0.37
	T-10.0	Not Sampled	-0.11	0.15, 0.26
C30-Control	T-0.0	0.18, 0.05	1.36, 0.46	0.03, -0.08
	T-2.5	0.00, 0.00	-0.06, 0.16	-0.05, -0.06
	T-5.0	0.00, 0.00	0.45, 0.08	-0.21, -0.04
	T-7.5	Not Sampled	0.13	-0.19, -0.37
	T-10.0	Not Sampled	Not Sampled	0.00, 0.06
C50-Control	T-0.0	0.11, 0.12	0.48, 0.52	0.09, 0.01
	T-2.5	0.02, -0.02	0.08, 0.04	0.00, 0.01
	T-5.0	0.00, 0.00	0.04, -0.09	-0.07, 0.02
	T-7.5	Not Sampled	0.16	-0.41, 0.12
	T-10.0	Not Sampled	Not Sampled	0.02, -0.14
D10-Control	T-0.0	0.01, 0.05	Not Sampled	Not Sampled
	T-2.5	0.22, 0.24	Not Sampled	Not Sampled
	T-5.0	-0.12, 0.00	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	0.12, -0.02	Not Sampled	Not Sampled
	T-2.5	0.02, 0.02	Not Sampled	Not Sampled
	T-5.0	0.00, 0.16	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled

Table 7.3.1. Ozone concentration in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Ozone (mg/L)		
D50-Control	T-0.0	0.18, -0.02	Not Sampled	Not Sampled
	T-2.5	0.22, 0.00	Not Sampled	Not Sampled
	T-5.0	-0.44, 0.00	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
[1] Duplicate analyses conducted				

As discussed in Section 3.2, the lifetime of ozone in marine waters (that is, waters with high concentrations of bromide ion) is expected to be ~5 seconds. The measurements of ozone appeared to fluctuate considerably around zero. These results suggest that no or little ozone was in the samples at the time of analysis.

Table 7.3.2. TRO (milligrams per liter as Cl_2) concentration in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Bromine (mg/L as Cl_2)		
A10- Treatment	T-0.0	-0.13, -0.14[1]	0.06, 0.07	0.07, 0.01
	T-2.5	0.21, 0.43	2.74, 2.80	4.02, 4.07
	T-5.0	0.23, 0.26	2.39, 2.37	OR, OR
	T-7.5	Not Sampled	OR	OR, OR
	T-10.0	Not Sampled	OR[2], OR	OR,OR
A30- Treatment	T-0.0	0.00, 0.00	0.06, 0.04	0.02, 0.02
	T-2.5	0.08, -0.01	2.70, 2.78	3.62, 3.77
	T-5.0	0.20, 0.03	2.84, 2.15	OR, OR
	T-7.5	Not Sampled	OR, OR	OR, OR
	T-10.0	Not Sampled	OR, OR	OR, OR
A50- Treatment	T-0.0	-0.11, -0.02	0.06, 0.05	-0.09, 0.01
	T-2.5	0.15, 0.14	0.37, 0.39	0.32, 0.31
	T-5.0	0.24, 0.26	2.42, 2.39	2.68, 2.72
	T-7.5	Not Sampled	4.70, 4.62	4.53, 4.80
	T-10.0	Not Sampled	OR, OR	OR, OR
B10- Treatment	T-0.0	-0.08, 0.01	0.02, 0.00	-0.04, 0.00
	T-2.5	0.00, -0.03	0.57, 0.56	0.70, 0.59
	T-5.0	0.10, 0.01	OR, OR	2.90, 3.80
	T-7.5	Not Sampled	3.89, 3.94	4.83, 4.72
	T-10.0	Not Sampled	OR, OR	OR, OR
B30- Treatment	T-0.0	0.05, 0.02	0.01, -0.05	-0.01, 0.01
	T-2.5	0.03, 0.01	0.85, 0.84	1.00, 1.08
	T-5.0	0.02, 0.00	OR, OR	3.98, 3.96
	T-7.5	Not Sampled	4.40, 4.37	OR, OR
	T-10.0	Not Sampled	OR, OR	OR, OR
B50- Treatment	T-0.0	0.01, 0.03	-0.03, 0.03	-0.03, -0.04
	T-2.5	0.01, 0.00	0.63, 0.61	0.96, 1.04
	T-5.0	0.09, 0.17	OR, OR	4.14, 4.12
	T-7.5	Not Sampled	3.91, 3.96	OR, OR
	T-10.0	Not Sampled	OR, OR	OR, OR
C10-Control	T-0.0	-0.04, 0.03	-0.29, -0.32	0.00, 0.05
	T-2.5	-0.13, 0.07	0.01, -0.01	0.06, 0.06
	T-5.0	0.24, 0.00	-0.06	0.05, 0.08
	T-7.5	Not Sampled	0.00, -0.04	-0.11, -0.11
	T-10.0	Not Sampled	0.00, -0.01	-0.05, -0.02
C30-Control	T-0.0	0.18, 0.05	-0.39, -0.38	-0.01, -0.05
	T-2.5	0.00, 0.00	0.00, -0.05	0.12, 0.06
	T-5.0	0.00, 0.00	-0.08, -0.10	-0.02, 0.02
	T-7.5	Not Sampled	0.00, -0.06	-0.11, -0.12
	T-10.0	Not Sampled	0.00, 0.00	-0.06, -0.11

Table 7.3.2. TRO (milligrams per liter as Cl_2) concentration in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Bromine (mg/L as Cl_2)		
C50-Control	T-0.0	0.11, 0.12	-0.36, -0.38	0.03, -0.10
	T-2.5	0.02, -0.02	0.03, -0.11	0.03, 0.08
	T-5.0	0.00, 0.00	-0.11, -0.09	0.02, 0.03
	T-7.5	Not Sampled	-0.01, -0.11	-0.14, -0.13
	T-10.0	Not Sampled	0.00, 0.00	-0.02, -0.06
D10-Control	T-0.0	0.01, 0.05	Not Sampled	Not Sampled
	T-2.5	0.22, 0.24	Not Sampled	Not Sampled
	T-5.0	-0.12, 0.00	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	0.12, -0.02	Not Sampled	Not Sampled
	T-2.5	0.02, 0.02	Not Sampled	Not Sampled
	T-5.0	0.00, 0.16	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D50-Control	T-0.0	0.18, -0.02	Not Sampled	Not Sampled
	T-2.5	0.22, 0.00	Not Sampled	Not Sampled
	T-5.0	-0.44, 0.00	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
[1] Results for duplicate analyses				
[2] OR = Out of Range, > 5 mg/L as Cl_2				

The reaction of ozone with bromide ion results in the formation of hypobromous acid (HOBr, see Section 3.2 for more details on the chemistry). This is in equilibrium with hypobromite ion (OBr^-) with a pK_a of 8.8 (Haag and Hoigne, 1983). Therefore, the analysis of bromine, as reflected by the TRO measurement, provides a total concentration of bromine in mg/L of Br_2.

As was discussed in Section 3.2, ozone rapidly reacts with OBr^- to form Br^-; however, it does not react with HOBr. The pH of the ballast water in these experiments was variable but was approximately 7.5. Therefore, a substantial portion of the total bromine would be in the HOBr form and non-reactive with ozone. The results in Table 7.2.2 confirm this in that the concentration of bromine (or, more correctly, TRO) in all experiments increased with an increase in the time of ozonation. In experiments 2 and 3, where it is believed that more ozone was introduced into the ballast water as a result of better equipment operation, the concentration was over range (ca. 5 mg/L) in all samples after the T3, T4 and/or T5.

It is hard to speculate on the exact residual concentration on the ship; however, this high concentration may explain part of the observed effect on organisms where bromoform and toxicity increased in the WET tests.

7.3.2 Oxidation Reduction Potential.

One of the objectives of this study was to explore potential measurements that could eventually be used as monitoring and control functions. One such measurement was ORP. The data obtained for the three experiments are summarized in Table 7.3.2.1.

Table 7.3.2.1. Oxidation Reduction Potential (ORP), as measured using the laboratory electrode, of the samples in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).

Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		ORP (mV)		
A10- Treatment	T-0.0	129.5	77.1	71.6
	T-2.5	372.4	725.1	767.3
	T-5.0	718.9	774.3	761.6
	T-7.5	Not Sampled	781.7	782.1
	T-10.0	Not Sampled	789.5	794.9
A30- Treatment	T-0.0	140.2	69.4	75.6
	T-2.5	363.7	738.3	750.7
	T-5.0	738.6	782.6	785.1
	T-7.5	Not Sampled	793.2	791.7
	T-10.0	Not Sampled	796.4	788.2
A50- Treatment	T-0.0	136.8	72.5	95.7
	T-2.5	289.7	629.3	574.8
	T-5.0	753.0	792.0	713.9
	T-7.5	Not Sampled	787.4	785.5
	T-10.0	Not Sampled	797.5	793.2
B10- Treatment	T-0.0	115.7	74.3	89.3
	T-2.5	217.0	297.1	637.5
	T-5.0	385.7	748.2	754.2
	T-7.5	Not Sampled	774.7	781.4
	T-10.0	Not Sampled	784.7	793.2
B30- Treatment	T-0.0	144.6	77.0	92.6
	T-2.5	217.3	981.0	721.1
	T-5.0	506.6	765.6	774.6
	T-7.5	Not Sampled	776.2	786.3
	T-10.0	Not Sampled	785.5	798.7
B50- Treatment	T-0.0	162.2	75.7	95.8
	T-2.5	339.9	672.3	716.9
	T-5.0	495.6	762.6	772.9
	T-7.5	Not Sampled	779.0	790.9
	T-10.0	Not Sampled	793.9	799.0
C10-Control	T-0.0	201.3	97.3	108.0
	T-2.5	313.1	164.4	255.7
	T-5.0	379.1	244.4	293.9

Table 7.3.2.1. Oxidation Reduction Potential (ORP), as measured using the laboratory electrode, of the samples in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		ORP (mV)		
	T-7.5	Not Sampled	258.7	420.0
	T-10.0	Not Sampled	273.7	294.3
C30-Control	T-0.0	197.6	103.4	106.8
	T-2.5	312.1	193.4	256.8
	T-5.0	347.4	245.8	274.9
	T-7.5	Not Sampled	246.7	417.4
	T-10.0	Not Sampled	278.1	287.0
C50-Control	T-0.0	198.3	99.0	107.3
	T-2.5	292.2	183.7	253.9
	T-5.0	360.8	238.9	294.0
	T-7.5	Not Sampled	260.3	438.7
	T-10.0	Not Sampled	272.5	294.7
D10-Control	T-0.0	196.6	Not Sampled	Not Sampled
	T-2.5	308.9	Not Sampled	Not Sampled
	T-5.0	359.9	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	198.6	Not Sampled	Not Sampled
	T-2.5	308.9	Not Sampled	Not Sampled
	T-5.0	359.9	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D50-Control	T-0.0	200.2	Not Sampled	Not Sampled
	T-2.5	306.0	Not Sampled	Not Sampled
	T-5.0	358.3	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
[1] BMDL = Below Method Detection Limit				
[2] Detection limit = 2 μg/L bromate ion				
[3] Detection limit = 10 μg/L bromate ion				

Comparing the total bromine concentrations and ORP measurements suggests that similarities exist in their concentrations as a function of ozonation (correlations not shown). Therefore, it is likely that the ORP measurement is recording the total oxidation state of the solution (but see also Section 7.10.1). In the case of the solutions that are ozonated, this is reflected in the measurement of total bromine. In the control ballast tanks, which also showed an increase in ORP with time, there are no similar correlations and the exact reason for this minor increase in ORP with sampling time are not clear. It may be that the probes used to measure ORP electrochemically "carry over" high mV signals for a short period of time even when placed into non-oxidized seawater.

7.3.3 Bromate Ion.

The bromate ion results are summarized in Table 7.3.3.1. The results indicate that bromate ion was always below the method detection limit in all samples. When bromate ion was spiked into the treated samples in the laboratory, the spike was never recovered fully. This result indicates that the water had a bromate ion demand. The cause of this apparent demand is not understood. However, it may be related to the high concentration of "active" bromine in the samples.

Table 7.3.3.1. Bromate ion data in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).

Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Bromate Ion (µg/L)		
A10- Treatment	T-0.0	BMDL[1,2]	BMDL[3]	BMDL[3]
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
A30- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
A50- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
B10- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
B30- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
B50- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
C10-Control	T-0.0	BMDL	BMDL	BMDL

Table 7.3.3.1. Bromate ion data in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Bromate Ion (μg/L)		
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
C30-Control	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
C50-Control	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
D10-Control	T-0.0	BMDL	Not Sampled	Not Sampled
	T-2.5	BMDL	Not Sampled	Not Sampled
	T-5.0	BMDL	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	BMDL	Not Sampled	Not Sampled
	T-2.5	BMDL	Not Sampled	Not Sampled
	T-5.0	BMDL	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D50-Control	T-0.0	BMDL	Not Sampled	Not Sampled
	T-2.5	BMDL	Not Sampled	Not Sampled
	T-5.0	BMDL	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
[1] BMDL = Below Method Detection Limit				
[2] Detection limit = 2 μg/L bromate ion				
[3] Detection limit = 10 μg/L bromate ion				

7.3.4 Bromoform.

The data for the concentration of bromoform in the three experiments are summarized in Table 7.3.4.1. In every experiment, the concentration of $CHBr_3$ increased with time. In those samples where a direct comparison could be made (that is from one experiment to another at the same time point), it is clear that the concentration of $CHBr_3$ increased more in Experiment 1 than in either 2 or 3. For all three experiments, the DOC was around 1 mg/L. Therefore, the differences were not due to a change in DOC concentration. In Experiment 3, the ozone-loading rate (i.e., the concentration or total ozone that was present) was higher than Experiment 1 or 2. The other variable that affects the amount of $CHBr_3$ formed is water temperature. It appears that this is the reason for the lower concentration of $CHBr_3$ in the two experiments that were conducted in November.

In general, three variables, the total amount of ozone delivered, DOC, and water temperature will affect the concentration of $CHBr_3$ that is formed. It is likely that ozonated water in the ballast tank upon standing (i.e., during the trip back to the port for a new cargo) will result in an increase in the concentration of $CHBr_3$. However, from the literature review, the concentration will not approach that, which would result in any toxicity to the receiving waters.

Table 7.3.4.1. Bromoform data in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).

Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Bromoform (µg/L)		
A10- Treatment	T-0.0	BMDL [1,2]	BMDL [2]	BMDL [2]
	T-2.5	35.0	62.0	74.6
	T-5.0	136	77.4	77.7
	T-7.5	Not Sampled	91.2	93.0
	T-10.0	Not Sampled	92.2	90.1
A30- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	30.0	68.4	80.0
	T-5.0	145	76.0	90.3
	T-7.5	Not Sampled	94.0	94.7
	T-10.0	Not Sampled	98.0	105.6
A50- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	104	35.1	29.3
	T-5.0	Not Sampled	75.2	75.2
	T-7.5	Not Sampled	80.3	94.6
	T-10.0	Not Sampled	82.4	96.1
B10- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	32.9	42.5
	T-5.0	24.0	53.8	73.7
	T-7.5	Not Sampled	73.6	96.5
	T-10.0	Not Sampled	76.1	107
B30- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	44.6 (44.7)[3]	55.5
	T-5.0	47.2	70.4	70.6

Table 7.3.4.1. Bromoform data in the ballast tank treated with ozone (columns A and B) and the control tank (C and D).				
Sample Location	Sample Time	Experiment 1	Experiment 2	Experiment 3
		Bromoform (μg/L)		
	T-7.5	Not Sampled	75.7	96.5
	T-10.0	Not Sampled	83.0	103
B50- Treatment	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	40.4	46.2
	T-5.0	35.8	58.7	87.1
	T-7.5	Not Sampled	74.8	79.0
	T-10.0	Not Sampled	79.4	105
C10-Control	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL(BMDL)	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
C30-Control	T-0.0	BMDL	BMDL	BMDL
	T-2.5	Not Sampled	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL(BMDL)	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
C50-Control	T-0.0	BMDL	BMDL	BMDL
	T-2.5	BMDL	BMDL	BMDL
	T-5.0	BMDL	BMDL	BMDL
	T-7.5	Not Sampled	BMDL	BMDL
	T-10.0	Not Sampled	BMDL	BMDL
D10-Control	T-0.0	BMDL	Not Sampled	Not Sampled
	T-2.5	BMDL	Not Sampled	Not Sampled
	T-5.0	BMDL	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	BMDL	Not Sampled	Not Sampled
	T-2.5	BMDL	Not Sampled	Not Sampled
	T-5.0	BMDL	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D50-Control	T-0.0	BMDL	Not Sampled	Not Sampled
	T-2.5	BMDL	Not Sampled	Not Sampled
	T-5.0	BMDL	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
[1] BMDL = Below Method Detection Limit				
[2] Detection limit = 5 μg/L bromoform				

7.4 Bacteria

7.4.1 Culturable Heterotrophic Plate Counts of Treated and Control Ballast Water

Results of the heterotrophic plate count from the Phase 1 study are displayed in Tables 7.4.1.1 and 7.4.1.2. The counts displayed are from either the direct spread plate method or the membrane filtration method for each sample. The numbers presented are selected from the method that provided the best range of countable colonies for the sample. For example, for the ozonated seawater samples, the ozone treatment method was very effective in inactivating culturable heterotrophic bacteria. If 100 µl aliquots of treated seawater were inoculated onto the surface of Marine R2A Agar by the spread plate method, there would typically be no colonies that would grow. Therefore, the culturable microorganisms were concentrated by using a membrane filtration method so the sensitivity of the enumeration assay could be increased. The numbers shown in Tables 7.4.1.1 and 7.4.1.2 are an average of the plating performed in triplicate or duplicate for each diluted or original portion of the seawater sample.

Table 7.4.1.1 displays the number of colony forming units that were found in treated and untreated water samples collected from *S/T Tonsina* during September and November 2001. The number of culturable microorganisms was between 10^5 and 10^6 colony forming units per liter before the ballast water was ozonated and throughout the duration of the experiment in the control ballast tank. After ozonation, the number of viable organisms declined by the first 2.5 hour sample. The decline was much greater in Experiments 2 and 3, compared to Experiment 1. In Experiments 2 and 3, the number of bacteria declined to 10^2 and 10^3 colony forming units per liter in samples collected from column B and the 50 foot sample from column A in the ozonated ballast tank. Bacteria in the 10 and 30-foot sample in column A declined to 10^0 to 10^1 colony-forming units per liter.

Following 5.0 hours of treatment, the bacteria populations continued to decrease in experiments 2 and 3 to a number below the detection limit (5 colony-forming units per liter) and 40 colony-forming units per liter. One-third of the samples collected after 5.0 hours contained bacterial levels less than the level of detection. Samples collected at 7.5 and 10 hours contained very few viable cells, if any viable cells were found at all.

7.4.2 Culturable Heterotrophic Plate Counts for Treated and Untreated Ballast Water Stored for up to 35 Days

A small experiment was conducted with treated and untreated ballast water that was collected during Experiment 1 and Experiment 3. This seawater was placed in 10-L sterile carboys and returned to the University of Washington for storage at 10°C. Aliquots of water were removed from the carboys following 2, 7, and 35 days. In Experiment 1, the number of culturable bacteria was below the level of detection when the treated sample was analyzed following 2 days of storage. In Experiment 3, the length of storage was extended to 35 days. In this experiment, bacterial numbers in the control sample remained elevated throughout the entire period of storage, between 10^5 and 10^6 colony forming units. For the treated ballast water, the number of culturable bacteria remained below the level of detection, 3 colony forming units per liter, for samples analyzed following 2, 7, and 35 days of storage.

Table 7.4.1.1. Enumerations of culturable heterotrophic bacteria from treated and control *S/T Tonsina* ballast tanks.

		Colony forming units (CFU) / L		
Sample Location	Time	Experiment 1 9/01	Experiment 2 11/01	Experiment 3 11/01
A10- Treatment	T-0.0	4.70×10^{6}	1.30×10^{6}	4.10×10^{5}
	T-2.5	1.00×10^{4}	1.00×10^{1}	1.00×10^{1}
	T-5.0	$<3.00 \times 10^{3}$	4.00×10^{1}	5.00×10^{0} *
	T-7.5	Not Sampled	$<3.00 \times 10^{0}$	5.00×10^{0} *
	T-10.0	Not Sampled	$<3.00 \times 10^{0}$	$<5.00 \times 10^{0}$ *
A30- Treatment	T-0.0	2.70×10^{6}	9.20×10^{5}	2.40×10^{5}
	T-2.5	3.00×10^{3}	3.00×10^{1}	7.00×10^{0}
	T-5.0	$<3.00 \times 10^{3}$	3.00×10^{0}	$<5.00 \times 10^{0}$ *
	T-7.5	Not Sampled	3.00×10^{0}	$<5.00 \times 10^{0}$ *
	T-10.0	Not Sampled	$<3.00 \times 10^{0}$	5.00×10^{0} *
A50- Treatment	T-0.0	2.30×10^{6}	9.30×10^{5}	3.20×10^{5}
	T-2.5	$<3.00 \times 10^{3}$	5.80×10^{2}	6.00×10^{2}
	T-5.0	$<3.00 \times 10^{3}$	$<3.00 \times 10^{0}$	2.00×10^{1} *
	T-7.5	Not Sampled	1.00×10^{1}	$<5.00 \times 10^{0}$ *
	T-10.0	Not Sampled	$<3.00 \times 10^{0}$	5.00×10^{0} *
B10- Treatment	T-0.0	1.64×10^{7}	9.40×10^{5}	3.60×10^{5}
	T-2.5	1.09×10^{6}	9.00×10^{2}	1.20×10^{3}
	T-5.0	3.00×10^{3}	4.00×10^{1}	5.00×10^{0} *
	T-7.5	Not Sampled	1.00×10^{1}	$<5.00 \times 10^{0}$ *
	T-10.0	Not Sampled	1.00×10^{1}	$<5.00 \times 10^{0}$ *
B30- Treatment	T-0.0	3.20×10^{6}	8.70×10^{5}	3.20×10^{5}
	T-2.5	6.40×10^{5}	5.00×10^{2}	1.30×10^{3}
	T-5.0	$<3.00 \times 10^{3}$	3.00×10^{1}	7.00×10^{0}
	T-7.5	Not Sampled	$<3.00 \times 10^{0}$	5.00×10^{0} *
	T-10.0	Not Sampled	$<3.00 \times 10^{0}$	$<5.00 \times 10^{0}$ *
B50- Treatment	T-0.0	1.10×10^{6}	8.50×10^{5}	5.20×10^{5}
	T-2.5	2.40×10^{5}	3.00×10^{2}	1.10×10^{3}
	T-5.0	3.00×10^{3}	4.00×10^{1}	7.00×10^{0}
	T-7.5	Not Sampled	1.00×10^{1}	5.00×10^{0} *
	T-10.0	Not Sampled	$<3.00 \times 10^{0}$	$<5.00 \times 10^{0}$ *
C10-Control	T-0.0	2.30×10^{6}	1.10×10^{6}	7.00×10^{5}

Table 7.4.1.1. Enumerations of culturable heterotrophic bacteria from treated and control *S/T Tonsina* ballast tanks.				
		Colony forming units (CFU) / L		
Sample Location	Time	Experiment 1 9/01	Experiment 2 11/01	Experiment 3 11/01
	T-2.5	1.10×10^6	3.70×10^7	6.40×10^5
	T-5.0	6.00×10^5	8.40×10^5	7.20×10^5
	T-7.5	Not Sampled	7.90×10^5	6.70×10^5
	T-10.0	Not Sampled	7.60×10^5	6.20×10^5
C30-Control	T-0.0	1.70×10^6	7.70×10^5	2.30×10^5
	T-2.5	9.00×10^5	3.30×10^7	6.60×10^5
	T-5.0	8.00×10^5	7.90×10^5	5.70×10^5
	T-7.5	Not Sampled	7.70×10^5	6.00×10^5
	T-10.0	Not Sampled	7.40×10^5	6.30×10^5
C50-Control	T-0.0	9.00×10^5	7.60×10^5	3.20×10^5
	T-2.5	7.00×10^5	8.70×10^5	7.40×10^5
	T-5.0	5.00×10^5	8.90×10^5	6.60×10^5
	T-7.5	Not Sampled	7.80×10^5	6.70×10^5
	T-10.0	Not Sampled	8.80×10^5	7.60×10^5
D10-Control	T-0.0	9.00×10^5	Not Sampled	Not Sampled
	T-2.5	7.00×10^5	Not Sampled	Not Sampled
	T-5.0	8.00×10^5	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D30-Control	T-0.0	8.00×10^5	Not Sampled	Not Sampled
	T-2.5	5.00×10^5	Not Sampled	Not Sampled
	T-5.0	6.00×10^5	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
D50-Control	T-0.0	5.00×10^5	Not Sampled	Not Sampled
	T-2.5	5.00×10^5	Not Sampled	Not Sampled
	T-5.0	4.00×10^5	Not Sampled	Not Sampled
	T-7.5	Not Sampled	Not Sampled	Not Sampled
	T-10.0	Not Sampled	Not Sampled	Not Sampled
* = Sample enumerated in duplicate instead of triplicate				
- = Sample not analyzed.				

Table 7.4.1.2. Enumeration of culturable heterotrophic bacteria in treated and untreated *S/T Tonsina* ballast water following 2, 7 and 35 days of storage of the seawater after the end of the ship-board ozone treatment experiment.				
2 days of Storage		Colony forming units (CFU) / L		
Sample Location	Time	Experiment 1	Experiment 2	Experiment 3
Control	T3	1.20×10^{6}	Not Sampled	-
Treatment		$<3.00 \times 10^{3}$	Not Sampled	$<3.00 \times 10^{0}$
Control	T5	Not Sampled	Not Sampled	6.00×10^{5}
Treatment		Not Sampled	Not Sampled	$<3.00 \times 10^{0}$
7 days of Storage				
Sample Location	Time	Experiment 1	Experiment 2	Experiment 3
Control	T3	Not Sampled	Not Sampled	-
Treatment		Not Sampled	Not Sampled	$<3.00 \times 10^{0}$
Control	T5	Not Sampled	Not Sampled	4.60×10^{6}
Treatment		Not Sampled	Not Sampled	$<3.00 \times 10^{0}$
35 days of Storage				
Sample Location	Time	Experiment 1	Experiment 2	Experiment 3
Control	T3	Not Sampled	Not Sampled	-
Treatment		Not Sampled	Not Sampled	$<3.00 \times 10^{0}$
Control	T5	Not Sampled	Not Sampled	2.00×10^{6}
Treatment		Not Sampled	Not Sampled	$<3.00 \times 10^{0}$
- = Sample not analyzed.				

7.5 Zooplankton

In the 5-hour ozone exposure experiment conducted on 24 September, the average percent of animals alive was uniformly high (range: 94-97%) in pre-treatment samples (Table 7.5.1). Mortality after five hours was different for the two treatment columns: column A had 91% mortality, and column B showed 47% mortality.

While the September zooplankton assemblage was dominated by the calanoid copepod *Paracalanus* sp., it was quite diverse, with several other relatively numerous copepod taxa as well as numerous planktonic larvae of barnacles, polychaetes, and other animals. After 5 hours of ozone exposure, the poecilostomatoid copepod *Corycaeus anglicus* and large Cirripedia (barnacle) nauplii appeared to be particularly unaffected by the treatment. On the other hand, small calanoid copepod nauplii larvae appeared to experience relatively higher mortality than did other organisms.

Table 7.5.1. Results of September 24 zooplankton ozone mortality experiment (n = 3).

9/24/2001	Average Percent Alive	SD	Average Percent Moribund	SD	Average Percent Dead	SD
			Pre-treatment			
Column A-treatment	93.7	0.6	5.7	1.5	0.7	1.2
Column B-treatment	95.3	1.2	3.7	2.1	1.0	1.0
Column C-control	97.0	2.0	1.7	0.6	1.3	1.5
Column D-control	95.7	1.5	3.0	1.0	0.3	0.6
			5 hours treatment			
Column A-treatment	1.7	0.6	7.3	3.1	91.0	3.0
Column B-treatment	25.0	4.0	27.7	0.6	47.3	3.5
Column C-control	92.3	1.5	5.3	2.3	2.3	1.5
Column D-control	92.7	2.9	6.0	2.6	1.3	0.6

The November 2 10-hours experiment had results similar to the September experiment, in showing differential mortality at 5 hours between the two treated columns (Table 7.5.2). In contrast to the September experiment, survival was higher in column A than in column B. Mortality after 5 hours was also lower in this experiment than in the September experiment (20 % vs. 47 % in the "high survival" column, 66 % vs. 91 % in the "low survival" column). After 10 hours of treatment, the pattern of differential mortality between the treatment columns persisted, although mortality increased.

Table 7.5.2. Results of November 2 zooplankton ozone mortality experiment (n = 3).

11/02/2001	Average Percent Alive	SD	Average Percent Moribund	SD	Average Percent Dead	SD
			Pre-treatment			
Column A-treatment	96.3	1.2	3.0	0.0	0.7	1.2
Column B-treatment	93.7	1.5	4.0	1.7	0.3	0.6
Column C-control	97.3	2.1	1.3	1.2	1.7	2.1
			5-hours treatment			
Column A-treatment	40.3	3.2	39.7	8.5	20.0	6.2
Column B-treatment	13.7	2.5	20.0	6.0	66.3	8.5
Column C-control	97.7	1.5	2.3	1.5	0.0	0.0
			10-hours treatment			
Column A-treatment	13.7	1.5	19.3	8.7	67.0	9.6
Column B-treatment	1.7	1.2	1.0	1.0	97.3	2.1
Column C-control	94.3	3.8	5.0	3.6	0.7	0.6

In the November 4 experiment, differences in mortality between the two treatment columns was far less marked, and mortality appeared much higher at both treatment times than in the other experiments (Table 7.5.3).

In the November experiments, taxonomic diversity was much lower than in September, and the zooplankton assemblage was largely dominated by late juvenile stages of the calanoid copepod *Paracalanus* sp. Each plankton tow contained several specimens of the Asian calanoid copepod *Pseudodiaptomus marinus*. As none of these species were found in plankton tows from Port Angeles harbor taken both day and night, during the November 2 experiment, it was presumed that the individual organisms observed represented remnants of ballast water from the ship's last voyage to Long Beach harbor, where *P. marinus* has been introduced. This species, the harpacticoid copepod *Microsetella* sp., and nematode worms appeared to be relatively resistant to ozone treatment as compared to the *Paracalanus* sp.

Table 7.5.3. Results of November 4 zooplankton ozone mortality experiment (n = 3).

11/04/2001	Average Percent Alive	SD	Average Percent Moribund	SD	Average Percent Dead	SD
			Pre-treatment			
Column A-treatment	89.7	7.0	6.0	2.6	7.7	6.8
Column B treatment	94.7	2.5	2.3	1.5	3.0	1.0
Column C control	93.3	4.0	3.7	0.6	3.0	3.6
			5 hours treatment			
Column A-treatment	7.7	5.7	8.3	4.2	84.0	7.0
Column B-treatment	1.7	1.2	6.0	2.0	92.3	3.1
Column C-control	97.0	1.0	1.0	1.0	3.3	1.2
			10 hours treatment			
Column A-treatment	1.3	2.3	2.0	2.0	96.7	3.1
Column B-treatment	0.0	0.0	0.7	1.2	99.3	1.2
Column C-control	93.3	1.5	2.3	0.6	4.3	1.5

7.6 Phytoplankton

During both experiments, dinoflagellate populations exhibited sharp decreases in the ozone treatment tank relative to the control tank (Figures 7.6.1 and 7.6.2). For the 2 November experiment, samples collected 10 hours after ozone treatment contained 0-18% of the initial concentrations of dinoflagellates at column A (with concentration increasing with depth) and 0% of initial concentrations of dinoflagellates at column B. For the 4 November experiment, dinoflagellates were not detected at all in the ozone treatment tank, creating estimates of 0% of the initial concentrations remaining 10 hours post-treatment for all depths. In contrast, dinoflagellate concentrations did not exhibit any clear decline in the control tank in either experiment, ranging from 70 - 745% of the initial concentration after 10 hours.

Microflagellate concentrations exhibited a similar pattern between treatments (Tables 7.6.1 and 7.6.2). Ten hours after treatment, microflagellate concentrations declined between 1-30% in column A and between 2-7% in column B during the 2 November experiment. Interestingly, the smaller decline in column A was also observed for dinoflagellates during this experiment, suggesting a spatial variation in performance of ozone treatment within this experimental run. In contrast, no such spatial variation was evident for the second experiment on

4 November, and microflagellates declined to 1-4% of initial concentrations. For both dates, no appreciable decline was evident in the control tank for microflagellates.

In general, the results suggest ozone treatment has a very strong effect on vegetative cells of dinoflagellates and microflagellates. The observed decline is probably due to mortality, whereby the vegetative cells are simply destroyed by ozonation. Some portion of this decline could result from sedimentation, but we did not measure the possible accumulation of cells or resting stages at the bottom during this first phase of work. However, because sedimentation would also have occurred in the control tank, mortality from ozone exposure is still the most likely explanation for reduced populations densities of dinoflagellates and microflagellates in the treatment tank.

The results for diatoms are much more difficult to interpret. For the 2 November experiment, diatom concentrations varied from 17-135% of the initial concentrations after 10 hours in the ozone treatment tank. For the 4 November experiment, similar measures ranged from 20-120%. On both dates, no clear decline in abundance was observed in the control tank over the same time course.

Although the results for diatoms suggest that ozone treatment may be much less effective on these organisms compared to the other two groups, this likely represents a limitation of microscopic methods used during this phase of analyses. More specifically, unlike the dinoflagellates and microflagellates, diatoms are identified on the basis of the shape and patterns of their silica cell walls (frustules) that will not decompose quickly when exposed to ozone. Thus, although present in direct counts in relatively high numbers (following treatment), it is not possible to determine whether the diatoms counted were dead or alive with the method used.

Overall, the phytoplankton results show considerable promise for ozone treatment to remove phytoplankton from ballast water. Clearly more replication is required, under a range of conditions, to test how successful this approach may be. Furthermore, additional measures are needed in the next phase to test for possible accumulation of phytoplankton in bottom sediments and distinguish live from dead diatoms.

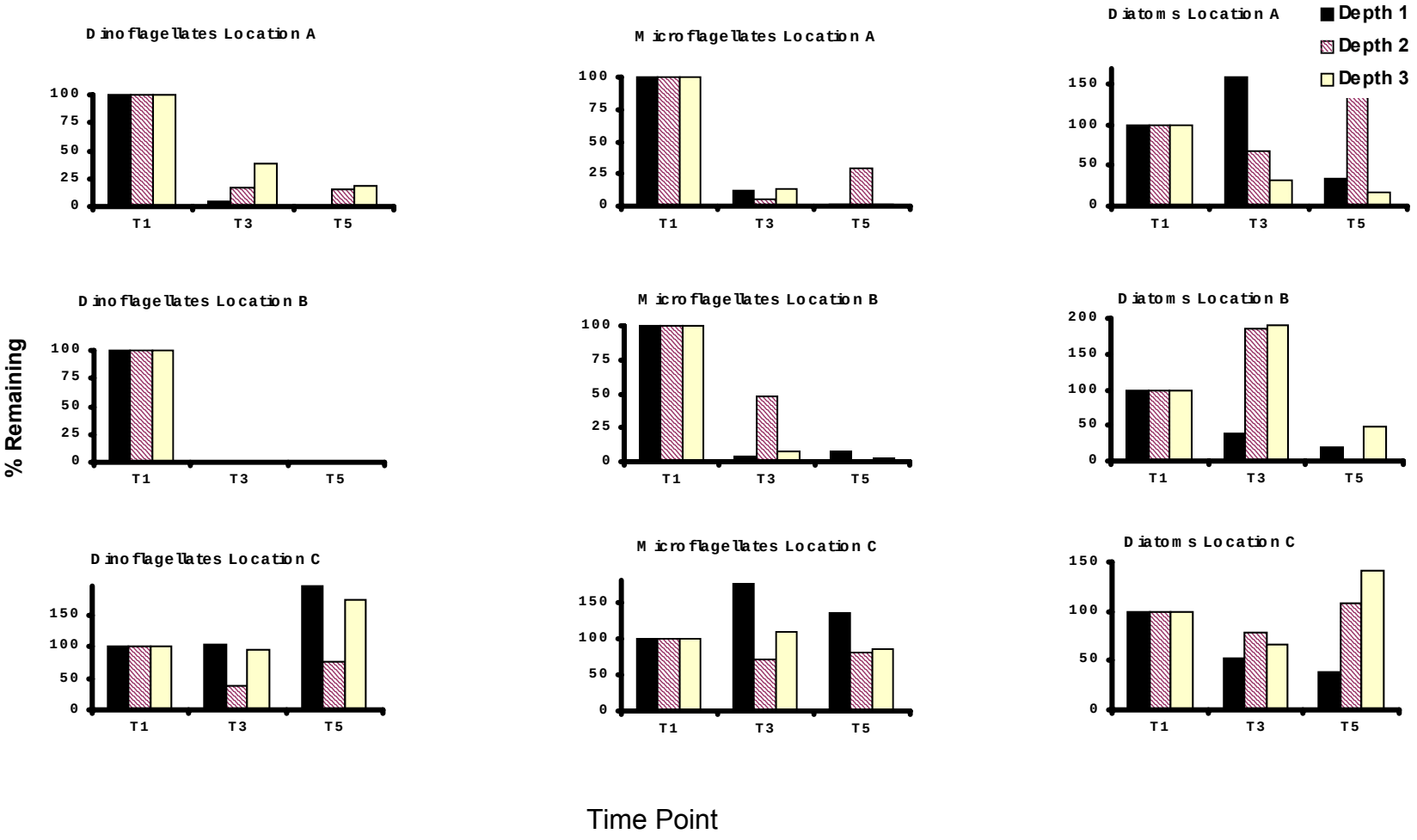


Figure 7.6.1. Change in concentrations of dinoflagellates, microflagellates, and diatoms during the ozone treatment experiment on 2 November 2001. Shown for each of 3 tank locations is the percentage of initial concentrations present at 3 depths. Percent change is relative to that measured at Time Point T1 for each tank and depth. Locations (i.e., columns) A and B were within the same tank that received ozone treatment, whereas location C was in a separate (control) tank, which did not receive ozone treatment. Samples at Time Point T1 were collected prior to ozone treatment, and Time Points T3 and T5 were collected at 5 and 10 hours, respectively, after ozone treatment was initiated. [Note: Sample lost for Location B, Time Point 5, Depth 2.]

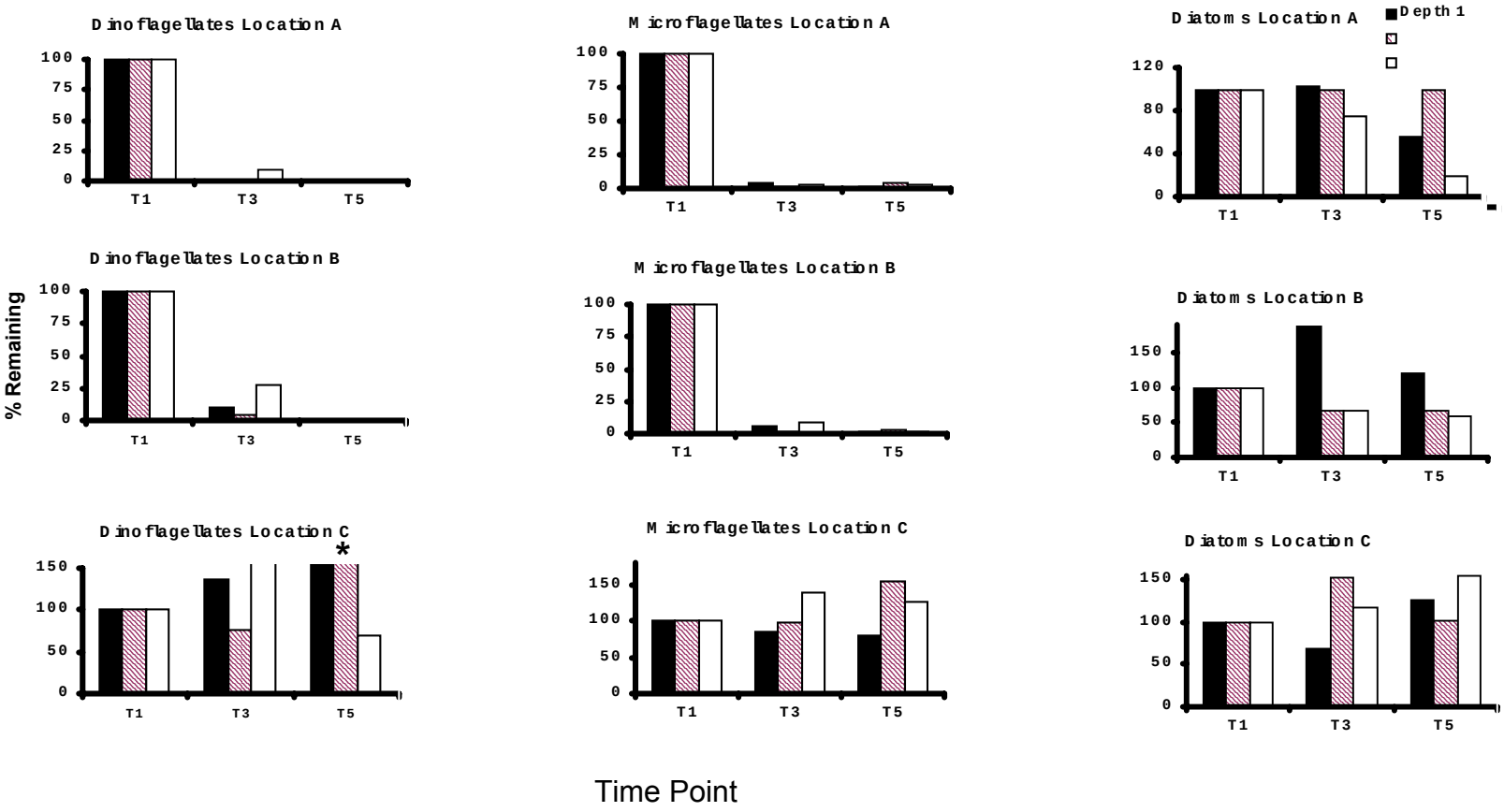


Figure 7.6.2. Change in concentrations of dinoflagellates, microflagellates, and diatoms during the ozone treatment experiment on 4 November 2001. Shown for each of 3 tank locations is the percentage of initial concentrations present at 3 depths. Percent change is relative to that measured at Time Point T1 for each tank and depth. Locations A and B were within the same tank that received ozone treatment, whereas location C was in a separate (control) tank, which did not receive ozone treatment. Samples at Time Point T1 were collected prior to ozone treatment, and Time Points T3 and T5 were collected at 5 and 10 hours, respectively, after ozone treatment was initiated. Asterisk indicates values that exceed the y-axis.

7.7 Caged Organism Experiments

7.7.1 Experiment 1

The first of three experiments exposed caged organisms in two ozone-treated columns and in two control columns for a 5-hour duration. Survival of control organisms was essentially 100 % (only 1 of 30 amphipods died, but 3 exposure chambers in Column C were lost) (Table 7.7.1.1). Survival was also 100% in ozone treated ballast tank Column B (aft column) for all species. In ozone Column A (fore column), mysid survival ranged from 20-60 % and sheepshead survival ranged from 0-30 %. Survival for both of these species was directly related to depth; those closest to the bottom suffered the highest mortality (Fig 7.7.1.1). Most of the surviving sheepshead and mysids were moribund (Table 7.7.1.1).

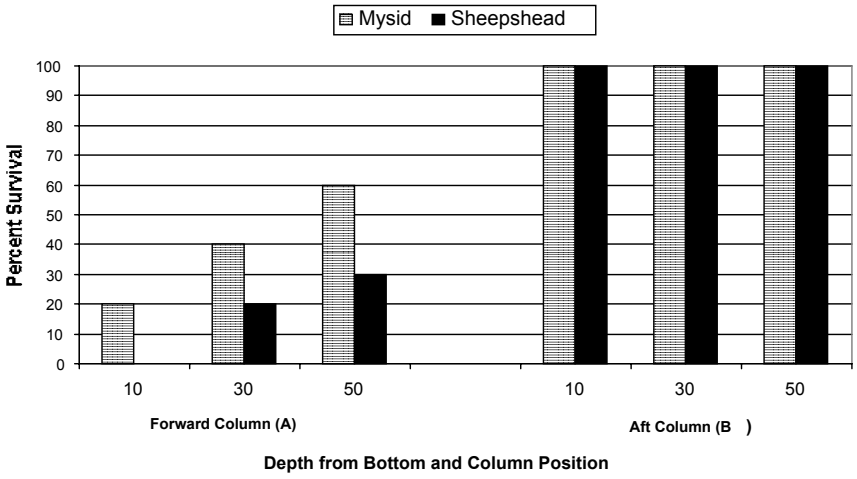


Figure 7.7.1.1. Survival of caged organisms in the forward ozone exposure column (A) during the first experiment (5-hour exposure).

Results of this first experiment suggested that: 1) the selected species showed a range in sensitivity to ozone, 2) toxicity was apparent in the forward ozone column (A), but absent in the aft column (B), and 3) toxicity may vary with depth in the water column. These observations suggested that a longer ozonation exposure time is necessary (or a higher ozone concentration is needed for the same exposure time) to affect high mortality for these species, and that the ozone system did not provide uniform ozone distribution laterally or vertically within the ballast tanks.

Table 7.7.1.1. Experiment 1 caged organism percent survival and percent moribund after 5 hours of ozone or control exposure.

		Percent Survival at 5 Hours				Percent Moribund at 5 Hours			
Treatment/Column	Depth*	Mysid	Sheepshead	Shore Crab	Amphipod	Mysid	Sheepshead	Shore Crab	Amphipod
Ozone A (fore)	0				100				0
"	0				100				0
"	0				100				0
"	10	20	0	100		0	All dead	0	
"	30	40	20	100		100	100	0	
"	50	60	30	100		83.3	33.3	0	
Ozone B (aft)	0				100				0
"	0				100				0
"	0				100				0
"	10	100	100	100		0	0	0	
"	30	100	100	100		0	0	0	
"	50	100	100	100		0	0	0	
Control (fore)	0				Chambers lost				Chambers lost
"	0				Chambers lost				Chambers lost
"	0				Chambers lost				Chambers lost
"	10	100	100	100		0	0	0	
"	30	100	100	100		0	0	0	
"	50	100	100	100		0	0	0	
Control D (aft)	0				100				0
"	0				90				0
"	0				100				0
"	10	100	100	100		0	0	0	
"	30	100	100	100		0	0	0	
"	50	100	100	100		0	0	0	

* Depth = distance in feet from the bottom of the tank

7.7.2 Experiment 2

The second experiment exposed all test organisms to a 10-hour ozonation duration in three treatment columns (fore, middle and aft) and to control conditions in only one column. For this experiment, control survival was essentially 100% for all species (only 1 of 30 mysids died), and none showed signs of any adverse effects (Table 7.7.2.1). For animals exposed to ozone in the three treatment columns, average percent survival by species was: mysid 51.4%, sheepshead 12.2%, shore crab 100.0%, and amphipod 92.2%. Once again, survival was a function of depth in the water column. Survival was the highest at the 10-foot station (closest station to the bottom), and decreased higher in the water column (30 and 50 foot stations; Figure 7.7.2.1; Table 7.7.2.1). This survival pattern, relative to depth, is opposite of that seen in Experiment 1. Degree of survival was also a function of column location: survival was lowest (and essentially equal) in the fore and middle columns, and was greatest in the aft column (Figure 7.7.2.1). In Experiment 2, all shore crabs survived and amphipods suffered only slight mortality (Figure 7.7.2.1). Of the surviving animals, the average percentage moribund was: sheepshead 88%, mysid 10%, amphipod 16%, and shore crab 0% (Table 7.7.2.1).

Results of the second experiment helped to clarify the relative sensitivity of the four test species. Their relative rank in sensitivity to ozone is: sheepshead > mysids > amphipods > shore crabs (Figure 7.7.2.1). The lateral pattern of toxicity was the same for Experiments 1 and 2: toxicity was highest in the fore columns and least in the aft columns. However, the vertical pattern of toxicity was different between Experiments 1 and 2; toxicity was lowest near the bottom in Experiment 2, which was the opposite of Experiment 1. This difference in vertical toxicity between the first two experiments may be due to a change in the ozone concentration in the water column.

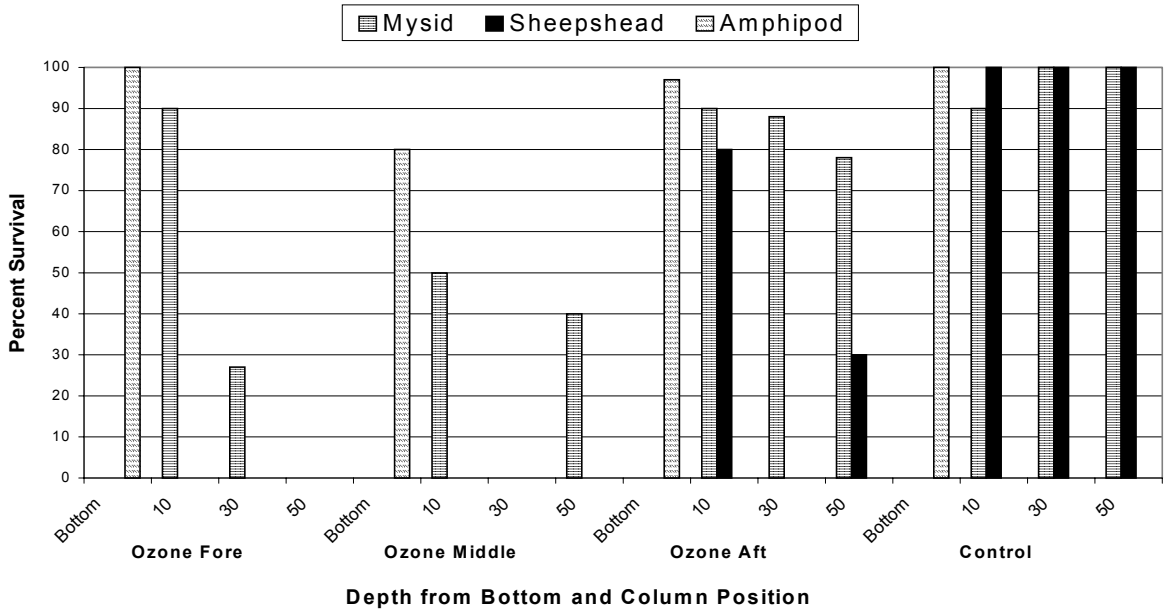


Figure 7.7.2.1. Average percent survival of caged organisms by depth and column position during the second experiment (10-hour exposure).

Table 7.7.2.1. Experiment 2 caged organism percent survival and percent moribund after 10 hours of ozone or control exposure.

		Percent Survival at 10 Hours				Percent Moribund at 10 Hours		
Treatment/Column	Depth*	Mysid	Sheepshead	Shore Crab	Amphipod	Mysid	Sheepshead	Shore Crab
Ozone 1 (fore)	0				100			
"	0				100			
"	0				100			
"	10	90	0	100		0	All dead	0
"	30	27	0	100		0	All dead	0
"	50	0	0	100		All dead	All dead	0
Ozone 4 (middle)	0				100			
"	0				90			
"	0				50			
"	10	50	0	100		0	All dead	0
"	30	0	0	100		All dead	All dead	0
"	50	40	0	10		0	All dead	0
Ozone 7 (aft)	0				100			
"	0				100			
"	0				90			
"	10	90	80	100		11.1	75	0
"	30	88	0	100		28.6	All dead	0
"	50	78	30	100		28.6	100	0
Control 3	0				100			
"	0				100			
"	0				100			
"	10	90	100	100		0	0	0
"	30	100	100	100		0	0	0
"	50	100	100	100		0	0	0

* Depth = distance in feet from the bottom of the tank

7.7.3 Experiment 3

The third experiment again exposed all test organisms for 10 hours in the fore, middle, and aft columns and to control conditions in one column. Control survival for this experiment was 100% and none of the control animals showed signs of stress (Table 7.7.3.1). For animals exposed to ozone, average percent survival by species was: mysid 31.1, sheepshead 0.0, shore crab 100.0 and amphipod 93.3. Many of the surviving mysids and about 15 % of the surviving amphipods appeared moribund (Table 7.7.3.1). Also, in the ozone exposure, all of the surviving shore crabs appeared to be moving sluggishly, a moribund state. For Experiment 3, there was no obvious trend in survival rates as a function of depth (Figure 7.7.3.1), although only mysids suffered partial kills, so data with which to make either vertical or lateral comparisons were sparse. As for the previous two experiments, survival (for mysids) was highest in the aft column. Once again, amphipods contained in the bottom buckets suffered only slight mortality (Table 7.7.3.1).

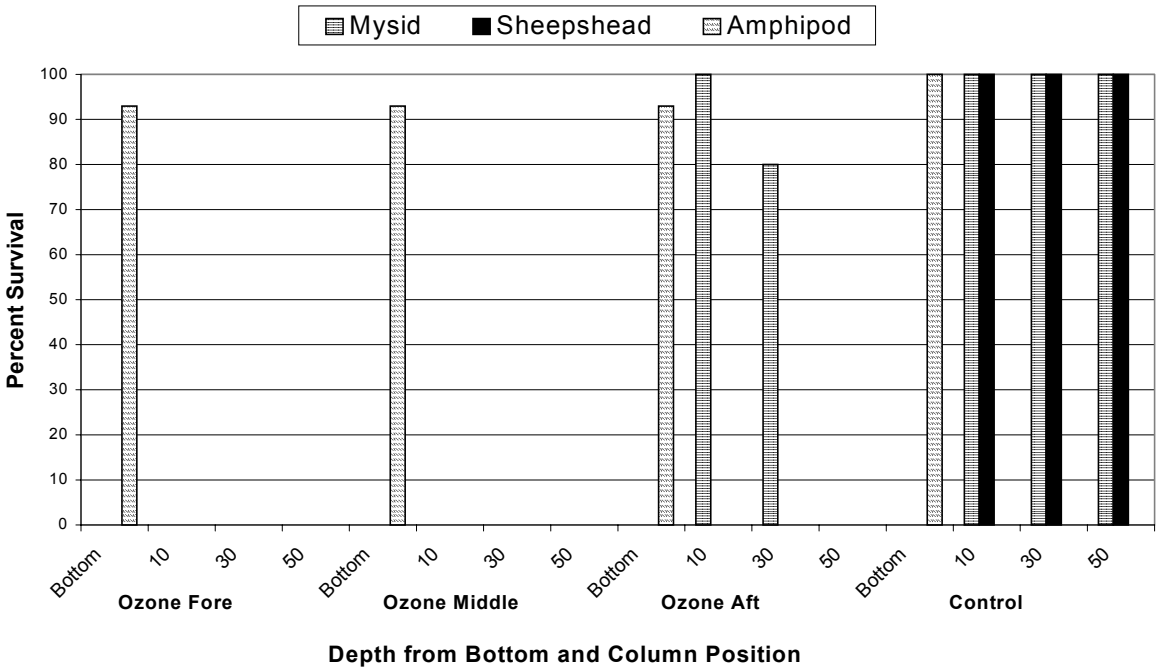


Figure 7.7.3.1. Average percent survival of caged organisms by depth and column position during the second experiment (10-hour exposure).

Table 7.7.3.1. Experiment 3 caged organism percent survival and percent moribund after 10 hours of ozone or control exposure.

		Percent Survival at 10 Hours				Percent Moribund at 10 Hours		
Treatment/Column	Depth*	Mysid	Sheepshead	Shore Crab	Amphipod	Mysid	Sheepshead	Shore Crab
Ozone 1 (fore)	0				100			
"	0				100			
"	0				80			
"	10	0	0	100		0	All dead	100
"	30	0	0	100		0	All dead	100
"	50	100	0	100		0	All dead	100
Ozone 4 (middle)	0				100			
"	0				100			
"	0				80			
"	10	0	0	100		0	All dead	100
"	30	0	0	100		0	All dead	100
"	50	0	0	100		0	All dead	100
Ozone 7 (aft)	0				100			
"	0				100			
"	0				80			
"	10	100	0	100		0	All dead	100
"	30	80	0	100		100	All dead	100
"	50	0	0	100		0	All dead	100
Control 3	0				100			
"	0				100			
"	0				100			
"	10	100	100	100		0	0	0
"	30	100	100	100		0	0	0
"	50	100	100	100		0	0	0

* Depth = distance in feet from the bottom of the tank

Starting with the most sensitive species, the results of Experiment 3 once again confirmed that the order of relative species sensitivity to ozone is sheepshead, mysids, amphipods, and finally shore crabs. Toxicity of ozone to the sheepshead and mysids was highest in Experiment 3. Also, differences in toxicity as a function of both depth and column position appeared to be less in this experiment, suggesting that increased ozone resulted in a more even distribution of the ozone and active bromine throughout the water column. Indeed, ozone loading rates in the vertical portion of the ozonated ballast tank (where the caged organisms were exposed) rose from 0.59 mg ozone/L/hour in Experiment 1 to 0.86 and 1.35 mg ozone/L/hour in Experiments 2 and 3, respectively.

7.7.4 Correlations

Mysids, being of intermediate sensitivity, gave the best "partial kill" data with which to calculate correlation coefficients with water chemistry parameters measured during the test. Mortality data were only collected at the end of each test (at 5 hours for Experiment 1, 10 hours for Experiments 2 and 3), while "ozone measurements" (i.e., ozone, bromine, ORP) were collected at 2.5-hour intervals.

The highest correlation was between mysid mortality and ORP measured next to the cages with the Hydrolab probe (all other measurements were collected in columns adjacent to the caged organisms; Table 7.7.4.1). The next highest correlation with mysid mortality was generally with bromine concentration, followed by ORP measured with the laboratory probe. Ozone concentrations showed very little correlation with mysid mortality (Table 7.7.4.1).

Table 7.7.4.1. Correlation coefficients (r) between end-of-test mysid mortality and ozone, bromine and ORP measurements at 2.5-hour intervals.

	Sampling Hour			
Parameter	2.5	5	7.5	10
Ozone	-0.0750	-0.3282	-0.1889	0.1284
Bromine	0.6689	0.4049	0.6302	OR*
Lab Probe ORP	0.3756	0.4558	0.1805	-0.1752
Hydrolab ORP	0.7287	0.7429	0.6329	0.8178

*All measurements were "over range"

7.8 Ballast Water Exchange

7.8.1 Experiment 1 (May 2001)

To measure the effect of BWE, changes in the density of selected zooplankton were compared over time between the experimental exchange tank and the control tank. Organisms were selected that were of coastal origin and not easily confused with open ocean forms in an effort to prevent the input of target organisms during the open ocean exchange process. Such input could partially compensate for declines due to exchange, thus reducing the accuracy of exchange measures. In addition, target organisms were selected based upon abundance, as well as organisms with initial densities greater than 50 m^{-3}. This latter criterion was used to improve the resolution beyond that possible with organisms of low initial density.

Following BWE, the residual density of 5 target organisms was from 3-22 % of the initial density (Table 7.8.1.1). However, this does not indicate exchange efficacy from 78 – 97 % during this voyage because significant mortality also occurred in the control tank (residual densities of 21 – 49 %).

To control for mortality due to time (i.e., independent of ozone treatment effects), the percent of residual organisms was compared between the two tanks and estimated exchange efficacy for each target taxon as:

[(Residual in Control Tank) – (Residual in Exchange Tank)] / (Residual in Control Tank)

Using this approach, exchange efficiency varied from 25 – 85% for the target taxa, including decapods, spionid polychaetes, cladocerans, gastropods, and barnacles. The mean value for these organisms was 59.05 with a standard error of 10.97 %.

Table 7.8.1.1. Effect of 100 % BWE on target organisms, Exchange Experiment I (May 2001). Shown are the concentrations (#/m^3) of selected organisms at two locations for an experimental exchange tank and control tank, collected at two time points (Time 0 and 2, before and after exchange respectively). Efficacy of exchange is estimated per target organism as: [(Mean % Change Control Tank) – (Mean % Change Exchange Tank)/ (Mean % Change Control Tank)] x 100.

Control Tank

	Location C			Location D			Summary		
	Time 0 (#/m^3)	Time 2 (#/m^3)	% Change	Time 0 (#/m^3)	Time 2 (#/m^3)	% Change	Mean % Change	SE	Mean % Residual
Decapoda	77.02	22.66	70.57	53.12	7.55	85.78	78.18	7.60	21.82
Spionidae	105.35	60.43	42.63	111.54	44.38	60.21	51.42	8.79	48.58
Cladocera	5141.64	846.08	83.54	1692.63	532.58	68.54	76.04	7.50	23.96
Gastropoda	2167.14	434.37	79.96	1324.36	506.14	61.78	70.87	9.09	29.13
Cirripedia	2974.50	426.82	85.65	1267.71	419.26	66.93	76.29	9.36	23.71

Exchange Tank

	Location A			Location B			Summary		
	Time 0	Time 2	% Change	Time 0	Time 2	% Change	Mean % Change	SE	Mean % Residual
Decapoda	84.10	2.83	96.63	93.84	2.83	96.98	96.81	0.17	3.19
Spionidae	99.15	10.20	89.71	55.77	17.94	67.83	78.77	10.94	21.23
Cladocera	3052.41	228.52	92.51	949.01	159.58	83.18	87.85	4.66	12.15
Gastropoda	856.94	168.08	80.39	757.79	184.14	75.70	78.04	2.34	21.96
Cirripedia	1189.80	46.27	96.11	573.65	33.05	94.24	95.17	0.94	4.83

Exchange Efficacy

	Difference (Control - Exchange)	% Efficacy (Difference/Control)
Decapoda	18.63	85.4
Spionidae	27.35	56.3
Cladocera	11.81	49.3
Gastropoda	7.17	24.6
Cirripedia	18.89	79.7
Mean		59.05
SE		10.97

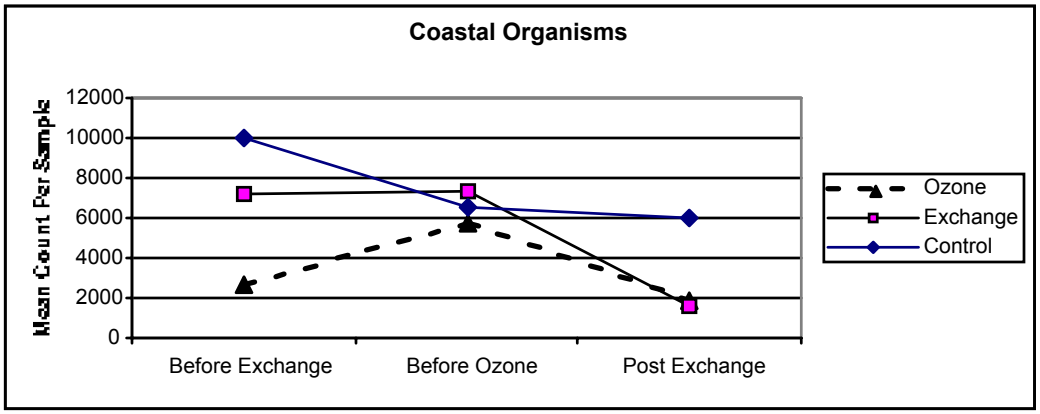
7.8.2 Experiment 2 (September 2001)

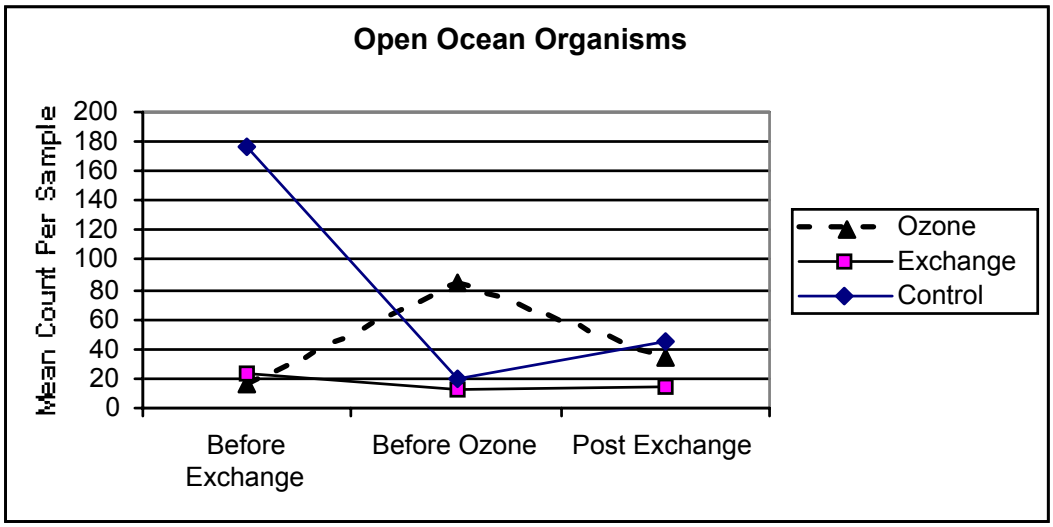
Planktonic organisms were ranked as to whether or not they were typical of coastal or oceanic habitats, or if they could have been from either habitat.

Samples were taken from all tanks at two pre-treatment time periods and one post-treatment time period. One may have expected the total abundance of organisms to be the same in a given tank in both pre-treatment periods, but this was not the case (Figure 7.8.2.1, Table 7.8.2.1 and 7.8.2.2). Total abundance of each type of organism was highest in the control tank, and lowest in the ozone treatment tank during the first pre-treatment sampling. Between the first and second pre-treatment samplings, abundance of coastal, ocean, and "other" organisms appeared to decrease in the control tank, stay about the same or decrease slightly in the exchange tank, and increase in the ozone treatment tank. Between the second pre-treatment sampling and post-treatment sampling, abundance of coastal and "other" species decreased in the ozone and exchange tanks but not in the control tank. The abundance of open ocean organisms stayed approximately the same. These results are reflected in the ANOVA results (Table 7.8.2.1) that indicated that not only were there highly significant time and tank effects, there were also significant habitat x time and time x tank interaction effects.

To explore the effects of treatment on specific "indicator" organisms, a set of four abundant coastal taxa were observed--cirripede (barnacle) nauplii, bivalve larvae, harpacticoid copepods, and polychaete annelid larvae. Except for harpacticoids, exchange and ozone treatment reduced these taxa, usually to near zero (Figure 7.8.2.2). For harpacticoids, numbers increased in exchange and control tanks, and decreased slightly in the ozone treatment tank. The reason for this increase in the exchange tank is unknown, but may have been due to the mixing of the bottom sediments and its associated fauna resulting from the exchange. ANOVA results for the selected coastal taxa showed significant reductions in polychaete and bivalve larvae, and no significant change for harpacticoids or cirripede nauplii, in both exchange and ozone treatment tanks (Table 7.8.2.2). However, as with combined organisms, there were usually significant time x tank effects, and starting numbers were quite different among the tanks.

One organism of particular interest, the Asian calanoid copepod *Pseudodiaptomus marinus*, was present in low numbers throughout the experiment. This species has not been reported north of California, where it has been introduced and established in several bays, and was presumably residual in the tanks from the ship's last port call in Long Beach Harbor. *P. marinus* was not found in several plankton tows that were made from the *S/T Tonsina* in Port Angeles Harbor at both day and night-time during the experiment. This may be an indication that the exchange that occurred in the open ocean between Long Beach Harbor and Port Angeles Harbor was not completely effective at removing this copepod. However, this organism was largely removed by the experimental exchange.





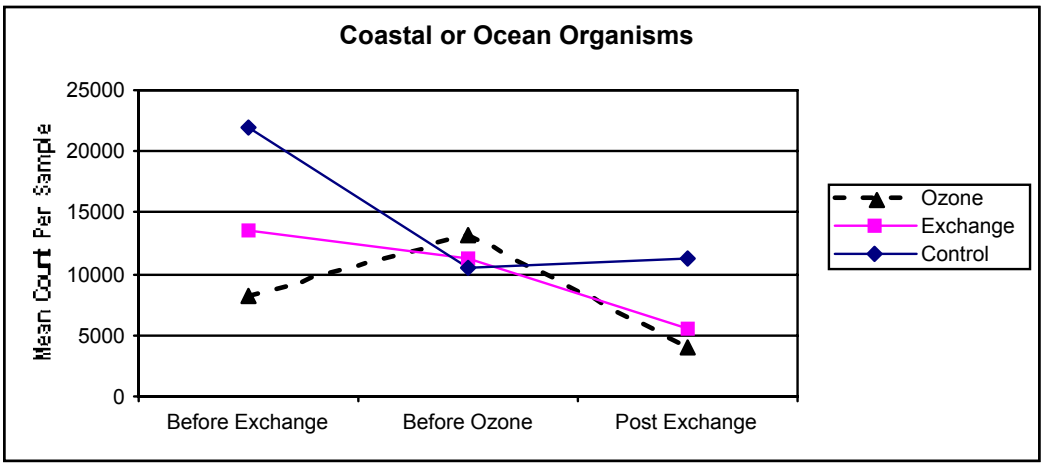


Figure 7.8.2.1. Numbers of planktonic organisms from three habitat categories during ballast exchange/ozone experiment.

Table 7.8.2.1. ANOVA results for all planktonic organisms from BWE/ozone experiment.

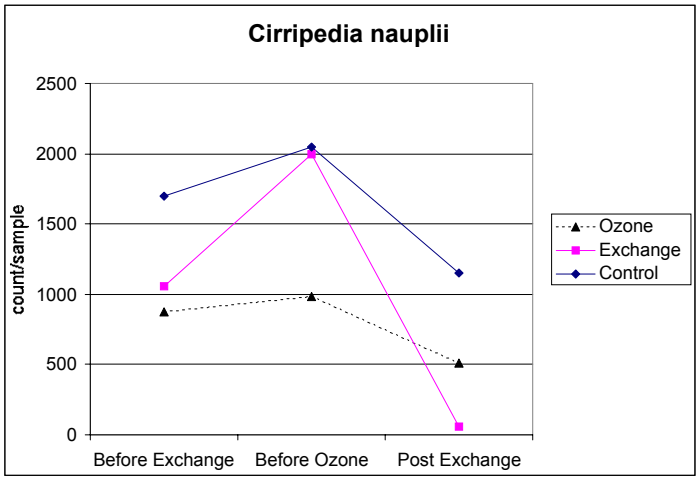
	HOV (p)	TIME (p)	order (smallest to largest)	TANK (p)	order (smallest to largest)	HABI-TAT (p)	order (smallest to largest)	HABI-TAT * TIME (p)	HABI-TAT * TANK (p)	TIME * TANK (p)	HABITAT * TIME * TANK (p)
log total organisms	0.00	0.000	3<2=1	0.000	O=E<C	0.000	0.000	0.014	0.134	0.000	0.405

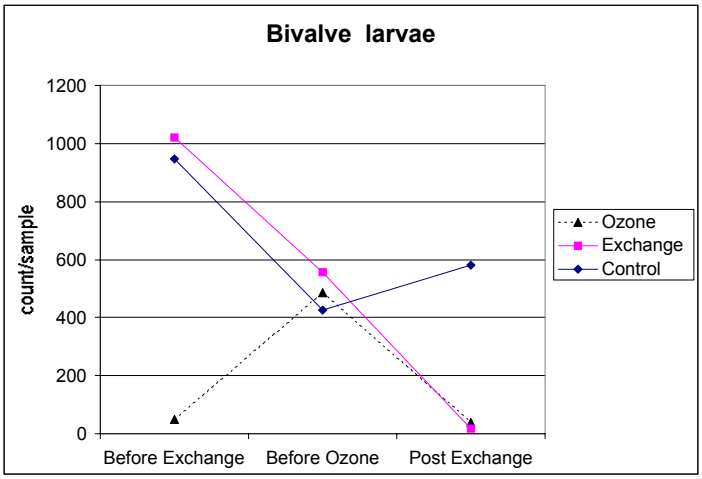
Log = log (count+1)
HOV = Homogeneity of variance by Levene's test
TIME: 1=Preexchange, 2 = Pre ozone, 3 = Post treatment
Tank: E = Exchange, O = Ozone, C = Control
Habitat: oc = ocean, c = coastal, ot = other
Order: rank with equalities based on Student Neuman Keuls Post Hoc Test

Table 7.8.2.2. ANOVA results for selected abundant coastal organisms from BWE/ozone experiment.

	HOV (p)	TIME (p)	Order (smallest to largest)	TANK (p)	Order (smallest to largest)	TIME * TANK (p)
log Cirripidea	**0.01**	**0.01**	3<2=1	0.13	E=O=C	**0.00**
log Polychaeta	**0.00**	**0.00**	3<1=2	**0.00**	O=E<C	**0.00**
log Bivalvia	0.23	**0.00**	3<2=1	**0.00**	O<E<C	**0.00**
log Harpacticoida	0.11	0.75	3=2=1	0.97	O=C=E	0.06

Log = log (count+1)
HOV = Homogeneity of variance by Levene's test
TIME: 1=Preexchange, 2 = Preozone, 3 = Post treatment
Tank: E = Exchange, O = Ozone, C = Control
Habitat: oc = ocean, c = coastal, ot = other
Order: rank with equalities based on Student Neuman Keuls Post Hoc Test





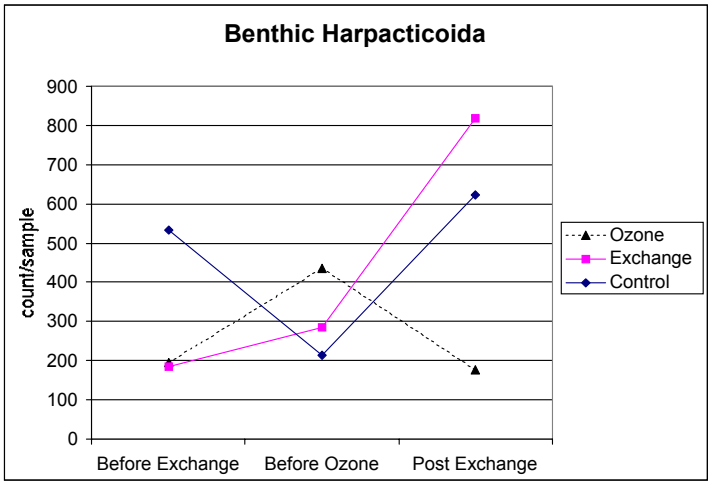


Figure 7.8.2.2. Numbers of four taxa of coastal planktonic organisms during ballast exchange/ozone experiment.

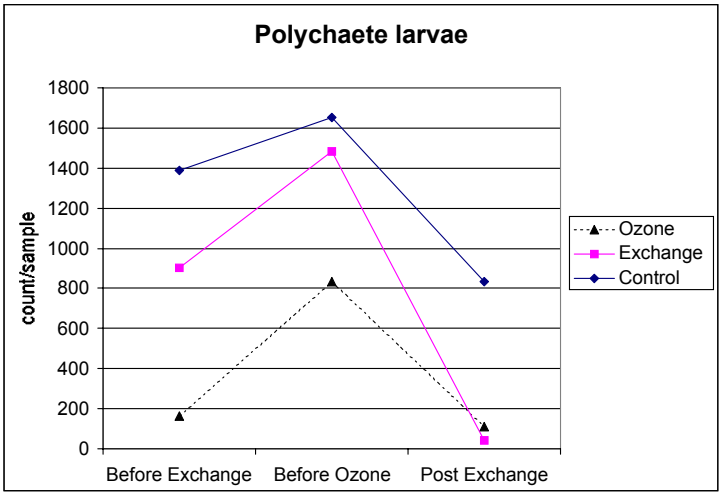


Figure 7.8.2.2 (continued). Numbers of four taxa of coastal planktonic organisms during ballast exchange/ozone experiment.

The mean exchange efficiency for Experiment 2, calculated as above, was 89% for the target taxa (Tables 7.8.2.3). This measure excludes the value for harpacticoid copepods, which occupy a different habitat (benthos versus plankton) and exhibited an increase following exchange (see above for possible explanation). The exchange efficiency for individual target taxa resident in the plankton ranged from 72.1% to 96.5%.

Table 7.8.3.1. Effect of 200 % BWE on Target Organisms, Exchange Experiment 2 (September 2001). Shown are the concentrations (#/m^3) of selected organisms at two locations for each an experimental exchange tank and control tank, collected at two time points (Time 0 and 2, before and after exchange respectively). Efficay of exchange is estimated per target organism as: [(Mean % Residual Control Tank) – (Mean % Residual Exchange Tank)/ (Mean % Control Tank)] x 100.

Control Tank

	Location C			Location D			Summary		
	Time 0 (#/m^3)	Time 2 (#/m^3)	% Change	Time 0 (#/m^3)	Time 2 (#/m^3)	% Change	Mean% Change	SE	Mean% Residual
Paracalanus sp.	991.00	581.50	41.32	1173.00	625.00	46.71	44.02	2.75	55.98
Harpacticoid sp.	147.00	48.00	67.34	119.00	263.50	121.14	54.36	60.25	154.36
Bivalvia	217.50	153.50	29.42	256.00	137.00	46.48	37.95	8.41	62.05
Cirrepedia	405.50	273.50	32.55	457.00	319.00	30.09	31.32	1.23	68.68
Podon sp.	69.50	40.00	42.44	79.00	43.00	45.56	44.00	1.56	56.00
Ploychaetes	338.00	209.50	38.02	357.00	202.00	43.42	40.72	2.50	59.28

Exchange Tank

	Location A			Location B			Summary		
	Time 0 (#/m^3)	Time 2 (#/m^3)	% Change	Time 0 (#/m^3)	Time 2 (#/m^3)	% Change	Mean% Change	SE	Mean% Residual
Paracalanus sp.	942.50	139.50	85.19	710.50	116.25	83.63	84.41	0.82	15.59
Harpacticoid sp.	61.50	135.00	119.51	41.00	273.75	567.68	343.60	136.54	343.58
Bivalvia	249.00	2.00	99.19	186.00	6.50	96.50	97.85	1.35	2.15
Cirrepedia	259.50	18.00	93.06	20.15	18.00	91.07	92.07	0.95	7.93
Podon sp.	51.00	2.00	96.07	33.00	0.00	100.00	98.04	1.95	1.96
Ploychaetes	224.50	15.00	93.31	162.00	7.50	95.37	94.34	1.03	5.66

Exchange Efficacy

	Difference value (Exchange-Control)		%Efficacy Difference value/ Control residual
Paracalanus sp.	40.39		72.15
Harpacticoid sp.	-289.69		NA
Bivalvia	59.89		96.51
Cirrepedia	61.05		88.89
Podon sp.	54.03		96.48
Ploychaetes	53.62		90.45
		Mean	88.89
		SE	4.46

7.8.3 Summary of Ballast Water Exchange Efficacy

There is good concordance in the percent efficacy of BWE measured in the two experiments. At first glance, it appears that efficacy was greater in Experiment 2 than Experiment 1 (89% versus 59%, respectively). However, the experiments differed in the amount of water exchanged. Experiment 1 measured the effect of a 100% exchange, whereas Experiment 2 measured the effect of a 200% exchange. When the results are standardized to estimate the effect of a 100% exchange, the mean efficacy for phytoplankton and zooplankton (excluding harpacticoid copepods, which are often associated with bottom communities) for the two experiments are similar: 59.1% and 69.1% (Table 7.8.3.1**)**. Thus, the mean efficacy of a 100% BWE between the two experiments was 64.1% (S.E. = 7 %).

The efficacy of exchange for the #4 tank aboard Tonsina appears lower than that measured, using a similar experimental approach, aboard other oil tankers along western North America (Ruiz et al., unpublished data). This result requires further replication to confirm. However, one possible explanation is that the efficacy of exchange is lower in double bottom tanks, due to internal structure and extensive "compartmentalization", compared to large wing tanks used for experiments aboard the other tankers.

If such variation among tanks is borne out by further measures (i.e., replicate experiments), this has important consequences for evaluation of performance for alternate ballast water treatments like ozone. Specifically, the relative value of alternate treatments is enhanced when exchange efficacy is low and when BWE is used as a standard for evaluation.

Table 7.8.3.1. Summary of BWE efficacy for Experiments 1 and 2. Shown for each experiment is the percent individual efficacy for each individual target taxon and the mean efficacy among taxa (with standard error; SE). The mean percent efficacy (and standard error; SE) is also shown for the two experiments combined, using the mean efficacy for each experiment.

A. Exchange Experiment 1 (May 2001)

	% Efficacy
Decapoda	85.4
Spionidae	56.3
Cladocera	49.3
Gastropoda	24.6
Cirripedia	79.7
Mean	59.05
SE	10.97

B. Exchange Experiment 2 (September 2001)

	% Efficacy
Paracalanus sp.	47.2
Harpacticoid sp.	NA
Bivalvia	81.4
Cirrepedia	66.7
Podon sp.	81.2
Ploychaetes	69.1
Mean	69.1
SE	13.99

C. Exchange Experiment 1 & 2: Pooled Results

	% Efficacy
Experiment I	59.05
Experiment II	69.10
Mean	64.08
SE	7.11

7.9 Whole Effluent Toxicity (WET) Tests

Because of the concern that toxic materials could be introduced into ballast waters as a result of ozonation, whole effluent toxicity (WET) tests were conducted on ozone-treated and untreated ballast water from three field trials. Tests conducted with non-treated ballast water samples (i.e., non-ozonated ballast water from the *S/T Tonsina*) exhibited no to minimal toxicity (i.e., less than 10% mortality) in any of the tests for both species. The median lethal concentrations were greater than100% ballast water, meaning that the non-treated ballast water showed no toxicity. Mysid shrimp median lethal concentrations in ozone-treated ballast waters ranged from approximately 50-70% ballast water, while topsmelt seemed to be slightly more sensitive exhibiting median lethal concentrations ranging from approximately 30-80% ballast water (Table 7.9.1). Results for Experiment 3 suggested that ozonated ballast water was more toxic (i.e., had lower median lethal concentrations) than in either of the first two experiments.

Table 7.9.1. Survival and median lethal concentration (EC50/LC50 as % ballast water) in acute toxicity tests conducted with mysid shrimp (*Americamysis bahia*) and topsmelt (*Atherinops affinis*) with post-ozonation ballast water samples collected on 24 September 2001 (Experiment 1), 2 November 2001 (Experiment 2), and 4 November 2001 (Experiment 3).

Test species / Exposure concentration (% ballast water)	% Survival		
	Experiment 1	Experiment 2	Experiment 3
Mysid shrimp (*Americamysis bahia*)			
0	100	90	97.5
6.25	95	100	97.5
12.5	100	95	97.5
25	100	95	75
50	100	95	0
100	0	0	0
EC50 (95% CI)	70.4 (69.5-71.3)	70.7 (50.0-100)	49.5 (27.0-37.7)
Topsmelt (*Atherinops affinis*)			
0	76*	100	100
6.25	80	95	95
12.5	88	100	100
25	92	100	80
50	100	47.5	0
100	20	7.5	0
LC50 (95% CI)	78.4 (71.1-86.5)	55.4 (47.8-63.1)	30.8 (28.1-33.9)

* Survival below minimum criteria for acceptable control survival.

7.10 Laboratory Ozone Toxicity Tests

Results of the WET tests with *A. bahia* and *A. affinis* indicated that one or more ozonation byproducts were stable enough to cause toxicity in ballast waters even 1-2 days after ozonation (Section 7.9). Further experiments addressing the toxicity and stability of these byproducts were conducted at ENSR's Fort Collins Environmental Toxicology Laboratory. The first experiment examined the possibility of continuing mortality after ozonation had been terminated and organisms were removed to clean seawater (post-exposure recovery test). Its purpose was to better define the toxicity of a relatively short-term (e.g., 1.5 hrs) ozone exposure on acute organism mortality over a 48-hour period. The second experiment was similar in design to the WET tests of ballast water samples. However, the ballast water WET tests had two acknowledged shortcomings: 1) oxidation-reduction potential (ORP) and total residual oxidant (TRO) measurements were not made at the time of sample collection or test setup, and 2) samples had to be shipped to the testing lab, causing a delay of 24-48 hours before tests could be initiated. Therefore, tests were initiated with ozonated waters that were 0-, 24-, or 48 hour-old and measured ORP and TRO at all significant time-points (latent toxicity test). Results of the latent toxicity test would either confirm or refute WET test results, and would allow for further definition of the dose-response relationship between stable byproducts (measured by TRO and ORP) and toxicity. Also, in a third experiment, it was determined whether the tubing placed in ballast tanks to collect water samples affected TRO or ORP measurements.

7.10.1 Ozone Chemistry

In a preliminary laboratory experiment, ORP and TRO provided contrasting information about the effect of Mud-Out® on ozone concentrations (Table 7.10.1.1). Measurements of TRO in the two aquaria were similar at 0.33 hours. In the aquarium with Mud-Out, TRO was higher at 0.66 and 1 hour; however, TRO was higher in the control aquarium at 2 and 3 hours. Differences were 0.6 and 0.4 mg/L at 1 and 2 hours. The difference increased to 1.6 mg/L at 3 hours. In contrast, ORP values in the two aquaria were within the error range of the electrode (± 20 mV) for all time points except 0.33 hour, when ORP in the aquarium with Mud-Out® exceeded that in the control aquarium by 63 mV.

Table 7.10.1.1. TRO and ORP values in aquaria with Mud-Out®.

Time (hours)		Control	Mud-out®
0.33	TRO (mg/L)	0.86	0.89
	ORP (mV)	646	709
0.66	TRO (mg/L)	2.28	3.04
	ORP (mV)	753	756
1.0	TRO (mg/L)	3.96	4.56
	ORP (mV)	765	770
2.0	TRO (mg/L)	6.64	6.25
	ORP (mV)	787	780
3.0	TRO (mg/L)	8.35	6.75
	ORP (mV)	792	787

The behavior of ORP and TRO over time was evaluated for multiple flows in subsequent organism exposures. Water chemistry data indicated that, in all exposures of organisms, ORP increased to approximately 700 mV after which only gradual increases were seen. In all

experiments, by 1 hour, ORP was greater than 700 at the two highest flows. However, no ORP measurement exceeded 795 mV, even when exposures lasted 5 hours. In contrast, TRO continued to increase with the distribution of ozone over time. An example of the general relationship between TRO and ORP seen across all experiments is illustrated in Figure 7.10.1.1.

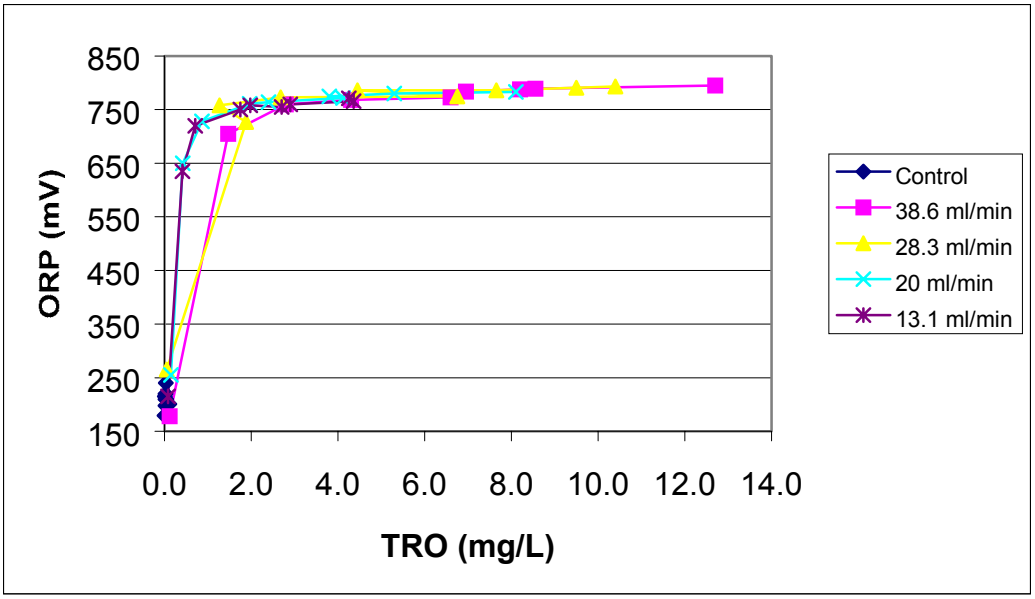


Figure 7.10.1.1. The relationship between TRO and ORP values during a typical 5-hour laboratory ozone exposure. Ozone flow rates are in ml/minutes.

7.10.2 Toxicity vs. ORP

Generally, organism mortalities dropped steeply when ORP levels approached the median effect concentrations (i.e., LC50 or EC50; see Figure 7.10.2.1 for an example) , regardless of ozone loading rates. The overall LC50 was lowest for *A. affinis* (Table 7.10.2.1). All *A. affinis* were dead at 2 hours in all aquaria receiving ozone. This time to complete mortality was the shortest for any of the species tested. Intermediate LC50 values were observed for *C. variegatus* and *A. bahia* (Table 7.10.2.1). All *A. bahia* in aquaria receiving ozone were dead at 3 hours; all but six *C. variegatus* in the lowest flow were dead at 3 hours, and these remaining six were dead at 4 hours. An EC50 was calculated for *R. abronius* based on motility (with prodding, if necessary). This value was similar to the LC50 of *C. variegatus* (Table 7.10.2.1). The experiment with *R. abronius* was terminated at 5 hours. Non-motile *R. abronius* were examined further for appendage movement in order to determine mortality. The overall EC50 was calculated using data through 4 hours of exposure. Finally, neither an EC50 nor LC50 could be calculated for *L. plumulosus* (Table 7.10.2.1). Exposure of *L. plumulosus* continued for 5 hours. Mortality did not exceed 30% in any aquarium. Across species, mortality commenced as ORP values approached and then exceeded 700 mV. The topsmelt, *A. affinis*, was the most sensitive species and the amphipod, *L. plumulosus*, was the least sensitive.

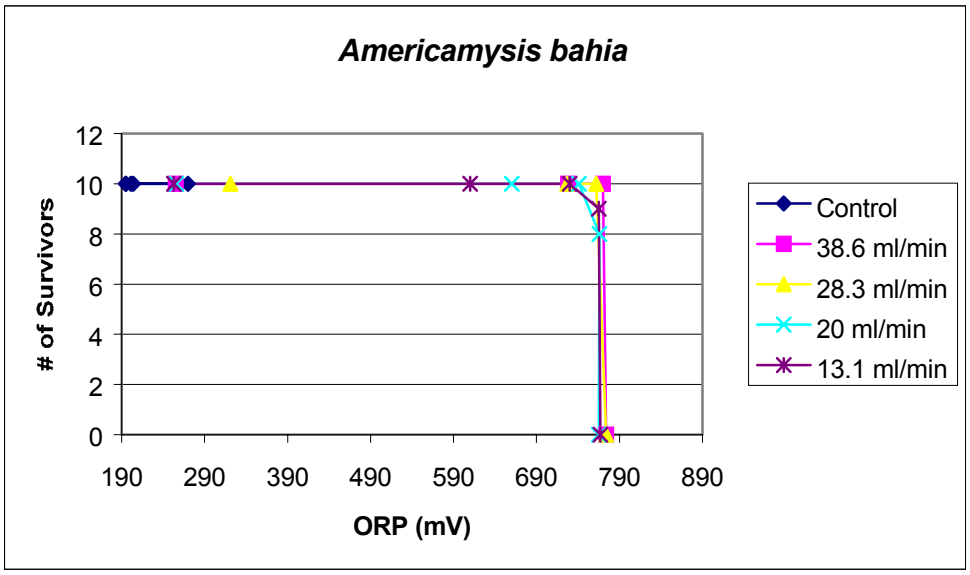


Figure 7.10.2.1. Typical relationship between ORP (mV) and survivorship. Ozone flow rates are in ml/minutes.

Table 7.10.2.1. LC50 or EC50 values for five species using ORP data.		
Species	LC50 or EC50 (mV)	95% Conf. Intervals (mV)
A. affinis	698	691, 705
A. bahia	768	767, 769
C. variegatus	746	741, 751
R. abronius	743	737, 750
L. plumulosus	> 795	n.a.

These data were consistent with results from the caged organism studies in which mortality (at least for mysids) also was strongly correlated to ORP measurements (Section 7.7). While specific effects thresholds (e.g., LC50) were not calculated for mysids in the caged studies, a plot of mortality as a function of ORP suggests a similar toxicity threshold would exist (Figure 7.10.2.2). Therefore, ORP measurements ranging from 700-800 mV appear to be associated with significant mortality in a variety of marine species both in the field and in the laboratory.

Furthermore, the relative sensitivity of test species exposed to ozone (as measured by ORP) was similar in both the field and lab experiments, with only limited exceptions. In the caged studies, the sheepshead minnow *C. variegatus* was the most sensitive species, followed by mysids (*A. bahia*) and amphipods (*R. abronius*). Of these species in the laboratory, LC50 values

for *C. variegatus* were indeed lower than *A. bahia*, suggesting that the sheepshead minnow was slightly more sensitive with respect to ORP exposure. One of amphipod species tested in the

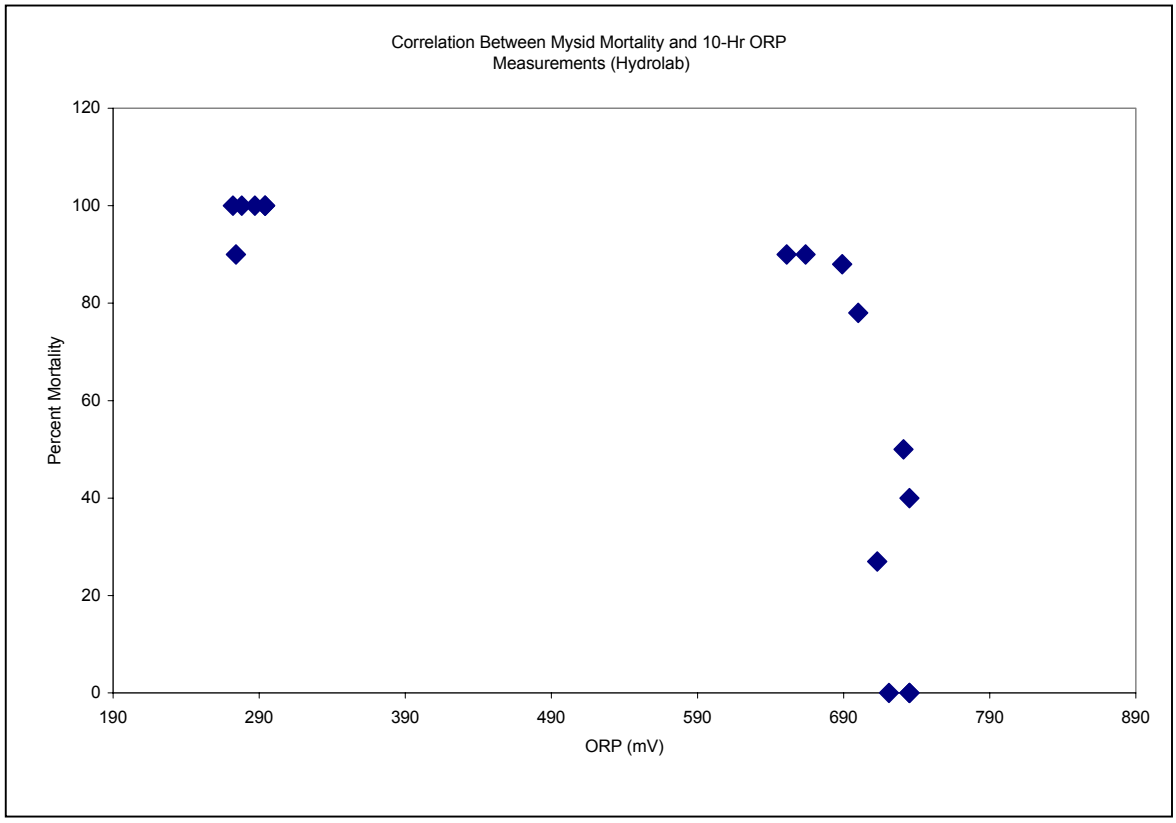


Figure 7.10.2.2. **Percent mortality as a function of ORP for *A. bahia* in caged studies.**

laboratory (*L. plumulosus*) was less sensitive to ORP than either sheepshead or mysids, but the second (*R. abronius*) exhibited similar sensitivity to sheepshead minnows.

7.10.3 Toxicity vs. TRO

Organism responses to ozonation were similar when evaluated against TRO vs. ORP measurements, although typical TRO dose-responses (Figure 7.10.3.1) did not exhibit as sharp a threshold as did ORP (Figure 7.10.2.1). The overall LC50 was again lowest for the topsmelt *A. affinis* (Table 7.10.3.1). Intermediate LC50 values were calculated for *C. variegatus* and *A. bahia* (Table 7.10.3.1), but the LC50 value for *A. bahia* differed from that of *C. variegatus* by > 1 mg/L. The EC50 calculated for *R. abronius* was not similar to the LC50 of *C. variegatus* (Table 7.10.3.1); using TRO data, this value was the highest calculated for any species. Neither an EC50 nor LC50 could be calculated for *L. plumulosus* (Table 7.10.3.1). Overall, these effects levels were approximately 2x-3x higher than similar values found in the toxicity literature (Section 3.3). However, most of the literature studies consist of longer-term (e.g., 48-96 hour) test durations. These longer experiments might require less TRO to induce the same level of effect as was observed in our short-term (3-5 hour) tests.

It is problematic to compare laboratory toxicity thresholds to zooplankton results from the field experiments (e.g., Section 7.5) because TRO (i.e., bromine) measurements often exceeded limits of detection using the Accu-vac vials (ca. 4 mg/L; see Table 7.3.2). However, with laboratory LC50 values ranging from 1.3 – 2.9 mg TRO/L (Table 7.10.3.1), this suggests that minimally-toxic TRO levels (i.e., levels inducing 50% mortality) could have been achieved on the *Tonsina* after as little as 2.5 hrs in Experiments 2 and 3 (Table 7.3.2).

Similar to ORP, the two fish species (*A. affinis*, and *C. variegatus*) were most sensitive to ozone toxicity when evaluated as a function of TRO (Table 7.10.3.1). This is also similar to the relative sensitivity to ozone exhibited by *C. variegatus* as a function of ORP in the caged studies (Section 7.7). Furthermore, the mean LC50 for *A. bahia* and the mean EC50 for *R. abronius* were greater than for either fish species, with the amphipod *R. abronius* being the least sensitive.

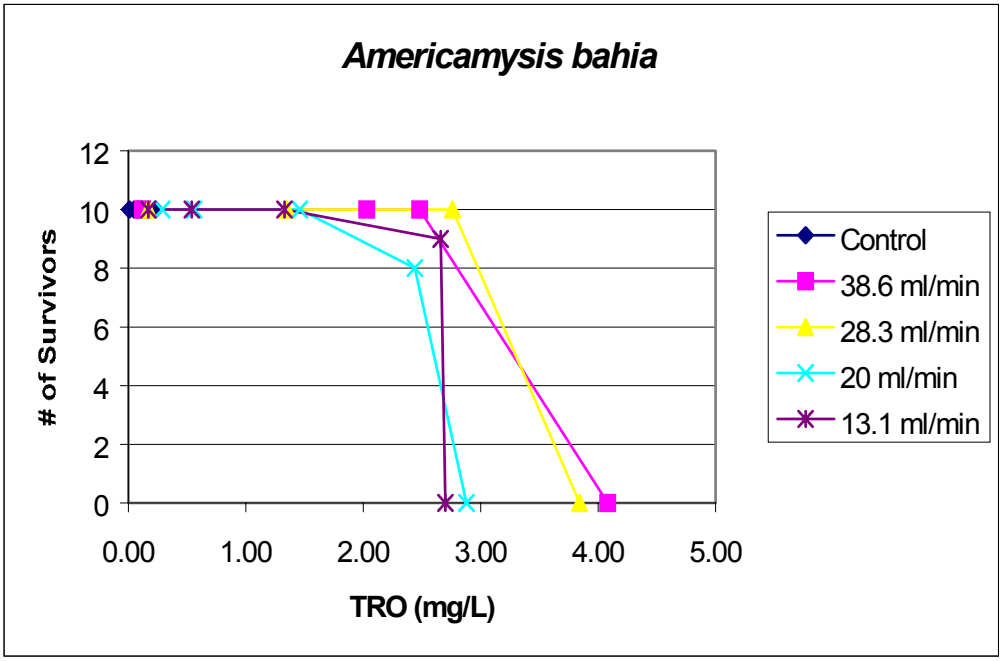


Figure 7.10.3.1. Typical relationship between TRO (mg/L) and survivorship. Ozone flow rates are given in ml/minutes.

Therefore, the relative sensitivity of marine organisms to ozone appears to be consistent between the caged and laboratory studies, with fish being the most sensitive, and amphipods being the least sensitive of the species tested.

Table 7.10.3.1. LC50 or EC50 values for five species using TRO data.		
Species	LC50 or EC50 (mg/L)	95% Conf. Intervals (mg/L)
A. affinis	1.29	1.11, 1.50
A. bahia	2.65	2.52, 2.80
C. variegatus	1.52	1.26, 1.84
R. abronius	2.93	2.49, 3.35
L. plumulosus	> 12.7	n.a.

7.10.4 Post-Exposure Recovery Test

After ozonation was terminated, biological effects of exposure were seen in *A. bahia* exposed to all ozone flows. Mortality occurred in the two highest flows at the time ozonation was terminated (0 hours, or immediately following termination of the ozone exposure) but all organisms were alive in the two lowest flows and in the control (Figure 7.10.4.1). All survivors from the highest flow (97.5 ml/minutes) and all but one from the 63.2 ml/minutes treatment were dead at 24 hours. Mortality was seen in the 20 ml/minute treatment at 24 hours. At 48 hours, all organisms were dead in the two highest treatments and mortality exceeded 50% in the two lowest treatments. No mortality occurred in organisms from the control aquarium at any time point.

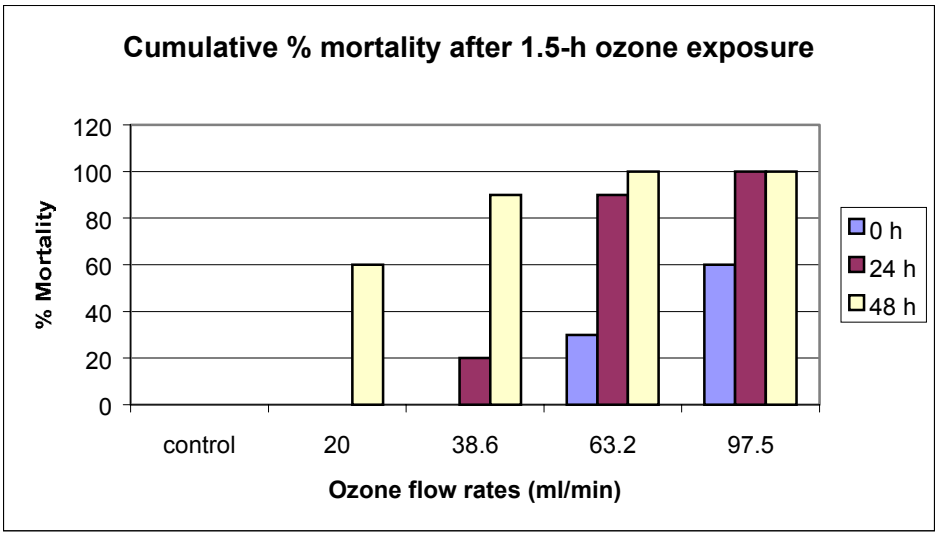


Figure 7.10.4.1. Cumulative mortality over 48 hours for *A. bahia* exposed to different flows of ozone.

Mortality data were also graphed against ORP and TRO values measured in the five aquaria at ozone termination (0 hours). ORP values in the aquaria receiving 38.6, 63.2, and 97.5 ml ozone/minute were within the error range of the electrode (± 20 mV). Initial mortality at 24 hours varied among these three treatments but, at 48 hours, mortality equaled either 90 or 100%

(Figure 7.10.4.2); the only *A. bahia* alive at 48 hours were moribund. Measurements of ORP in all treatment aquaria were ≥ 699 mV; mortality in organisms from treatment aquaria at 48 hours ranged from 60-100%.

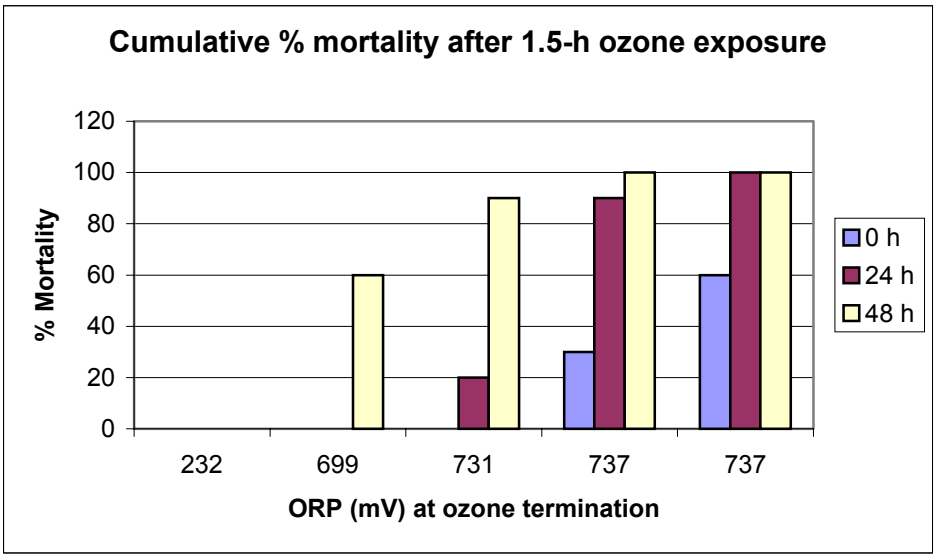


Figure 7.10.4.2. Relationship of ORP measurements to cumulative mortality for *A. bahia* exposed to ozone.

For TRO, initial mortality occurred in treatments where values exceeded 4.5 mg/L (Figure 7.10.4.3). In treatments where TRO was ≥ 4 mg/L, mortality occurred by 24 hours and was 90-100% by 48 hours; the only *A. bahia* alive at 48 hours was moribund. In the lowest ozone flow the TRO measurement at ozone termination was 0.7 mg/L. Mortality did not occur until 48 hours but then reached 60%; one of the four survivors was moribund.

Caged organism studies on the *S/T Tonsina* and laboratory studies have determined that *A. bahia* resist ozone toxicity at an intermediate level. They can survive ozone exposures of up to ten hours, though in reduced numbers. However, as noted for the experiments on the *Tonsina* (Section 7.7), many of the surviving *A. bahia* were moribund. Questions of possible delayed death and ecological relevance were raised with these results. The results of the current experiment support the hypothesis stated in the report that individuals exposed to ozone likely suffer damage and may succumb to it in subsequent days. While mortality was observed in only two ozone treatments at the termination of ozone exposure, and mortality in only one of those treatments exceeded 50%, mortality reached 60-100% at 48 hours post-exposure for all four ozone treatments.

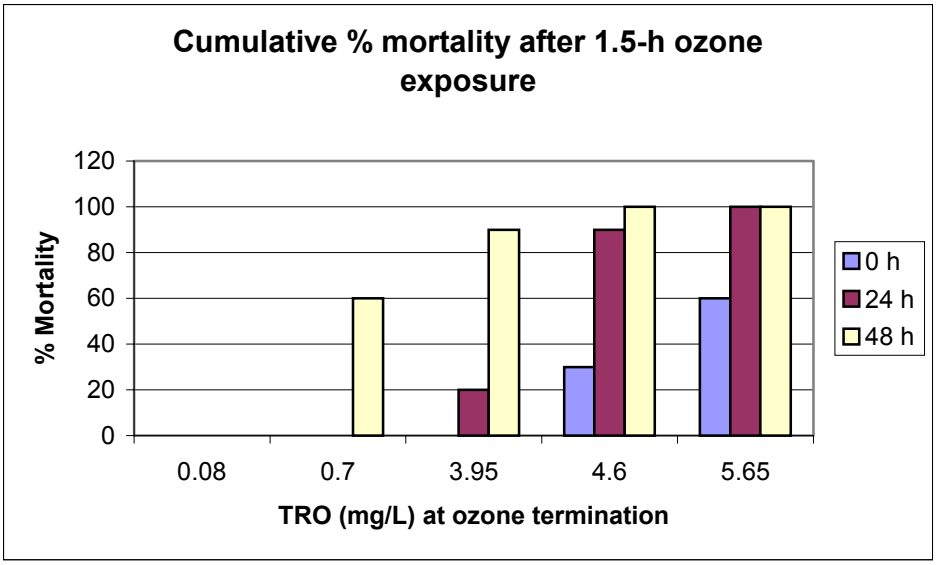


Figure 7.10.4.3. Relationship of TRO measurements to cumulative mortality for *A. bahia* exposed to ozone.

With data from previous laboratory studies of *A. bahia* (Section 7.10.2), an LC50 of 768 mV was calculated after 3 hours of ozone exposure. Final ORP measurements in all aquaria for the post-exposure recovery test were below this. However, previous studies on a variety of marine organisms conducted at the Fort Collins Environmental Toxicology Laboratory and on the *S/T Tonsina* found significant mortality associated with ORP measurements ranging from 700-800 mV. Final ORP measurements in all aquaria in the current study approached or were within this same range, even after only a relatively short (i.e., 1.5 hours) exposure.

ORP measurement is thought to reflect the total oxidation state of the solution, with total bromine (hypobromous acid and hypobromite ion) being a primary oxidant (Section 3.2). In caged studies on the *S/T Tonsina*, *A. bahia* mortality correlated most highly with ORP measured next to the cage and then with bromine concentration (Section 7.7.4), suggesting a relation of both to each other and to mortality. In this study, water chemistry measures suggest that oxidants, most probably total bromine, are causing mortality not only through exposure but also through a delayed physiological mechanism.

Further support for the association of mortality and oxidants was suggested by the TRO data. TRO, measured as TRC, likely consists mostly of bromine species in seawater (Section 7.3; Crecelius 1979). TRO measurements showed a greater difference between ozone loading treatments than ORP measurements. Mortality (30-60%) occurred within the 1.5 hours of ozone exposure in treatments where TRO exceeded 4.0 mg/L, and 100% mortality was observed in survivors by 48 hours. A TRO measurement less than 1.0 mg/L did not cause mortality within 1.5 hours or at 24 hours post-exposure, but was associated with 60% mortality at 48 hours post-exposure. Effects at this lower level of bromine have been cited in the literature, though primarily for freshwater species (Table 3.3.2). Therefore, it appears that sufficient amounts of

bromine oxidants may have built up in the ozonated water over 1.5 hours to have induced both immediate and delayed mortality (even up to 48 hours later) of *A. bahia*.

7.10.5 Latent Toxicity Tests

Mortality in the 100, 75, and 50% dilutions from 0-hour samples was 100% after 24 hours. Partial mortality (30%) was seen at 24 hours in 25% water, and increased at 48 hours (63%). An LC50 could not be calculated for the 0-hour samples because greater than 50% mortality occurred in all ozonated water mixtures at 48 hours. Therefore, the LC50 is reported as less than 25% ozonated water (Table 7.10.5.1). ORP and TRO values at the initiation of the experiment are listed in Table 7.10.5.2. Exact LC50 values also could not be calculated using these values for the reasons mentioned above (Table 7.10.5.1).

Table 7.10.5.1. 48-hour LC50 values for *A. bahia* in ozonated waters tested immediately after ozonation or held 24 or 48 hours before testing.

Test	% ozonated water	ORP (mV)	TRO (mg/L)
0-hr	< 25 %	< 704	< 1.32
24-hr	30	650	0.96
48-hr	26	704	0.71

Table 7.10.5.2. ORP and TRO values at the initiation of the 0-hour sample experiment.

Treatment	ORP (mV)	TRO (mg/L)
Control	190	0.05
25%	704	1.32
50%	738	2.48
75%	754	4.00
100%	761	5.05

Ozonated water held 24 hours also caused 100% mortality of *A. bahia* after they were exposed for 24 hours to 100, 75, and 50% waters. Partial mortality (10%) was seen in 25% water at 24 hours; this increased to 33% at 48 hours. The 48-hour LC50 was 30% ozonated water (Table 7.10.5.1). As a function of ORP and TRO data, 48-hour LC50 values were 650 mV and 0.96 mg/L. ORP and TRO values at the initiation of the experiment are listed in Table 7.10.5.3.

Table 7.10.5.3. ORP and TRO values at the initiation of the 24-hour sample experiment.		
Treatment	ORP (mV)	TRO (mg/L)
Control	218	0.09
25%	624	0.74
50%	724	1.93
75%	747	2.02
100%	759	4.80

Finally, ozonated water held 48 hours caused 100% mortality of *A. bahia* after they were exposed for 24 hours to 100, 75, and 50% waters. Partial mortality (20%) was seen in 25% water at 24 hours; this increased to 47% at 48 hours. The 48-hour LC50 was 26% ozonated water (Table 7.10.5.1). Based on ORP and TRO data, LC50 values were 704 mV and 0.71 mg/L. ORP and TRO values at the initiation of the experiment are listed in Table 7.10.5.4.

Table 7.10.5.4. ORP and TRO values at the initiation of the 48-h sample experiment.		
Treatment	ORP (mV)	TRO (mg/L)
Control	210	0.09
25%	702	0.67
50%	741	1.76
75%	751	3.20
100%	755	4.25

As noted in Tables 7.10.5.2 to 7.10.5.4, ORP measurements in 100% ozonated seawater stored for 0, 24, and 48 hours were similar within the error range of the electrode (± 20 mV) and, thus, were not decreasing over time in the sealed cubitainer. Similarly, measurements of TRO decreased over time, but only by ca. < 1 mg/L. This is consistent with these analyses (Table 7.10.5.1) that suggest the toxicity of ozone-produced oxidants generally remains constant regardless of how long samples were stored in closed cubitainers. However, it should be noted that once ozonated seawater was introduced to the open containers for biological testing, oxidant concentrations diminished relatively quickly. In general, ORP and TRO decreased over time after waters were added to the test beakers, which were not sealed (Figures 7.10.5.4 and 7.10.5.2).

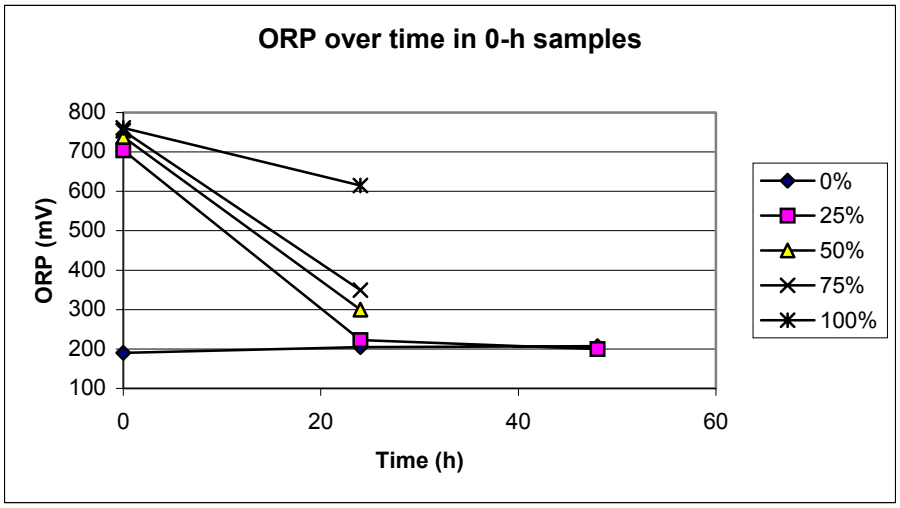


Figure 7.10.5.1. Change in ORP over time in 0-hour samples.

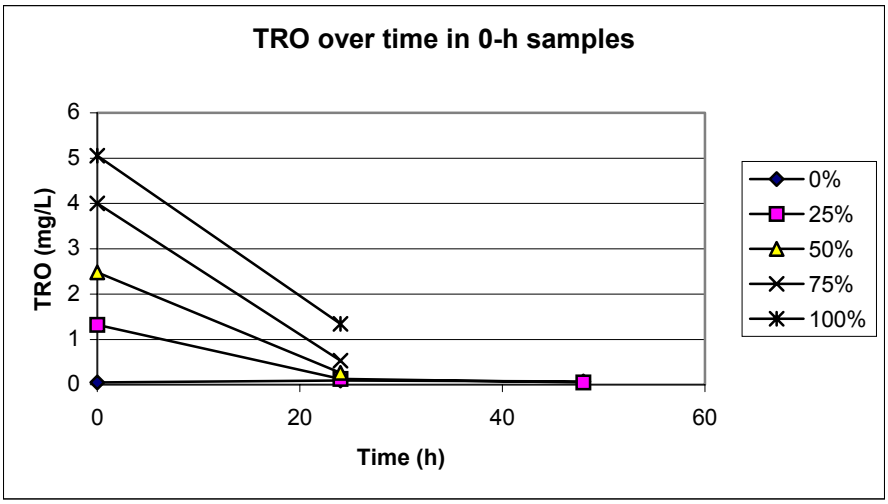


Figure 7.10.5.2. **Change in TRO over time in 0-hour samples**.

WET tests conducted with ballast water from experiments on the *S/T Tonsina* indicated that latent toxicity existed in the ozonated waters. One of the ozonation byproducts that is the most likely to be stable enough in storage under closed, cool conditions and still cause toxicity would be bromine (Section 3.3). No ORP or TRO measurements were taken during the WET tests. During our latent toxicity tests, however, ORP and TRO were measured at test initiation and at time points when biological data were collected.

The oxidants did not disappear from ozonated waters held 24 or 48 hours in a sealed container at 12 ºC. All organisms died when exposed to 50,75, or 100% ozonated water in tests initiated at any of the three time points (0 hours, 24 hours, 48 hours). ORP of the ozonated water

was 761 mV after ozonation was terminated and 755 mV 48 hours later. The corresponding TRO values were 5.05 and 4.25 mg/L. Thus, a stable oxidant appeared to exist in these waters, presumably bromine. ORP was > 720 mV and TRO > 1.76 mg/L in treatments where 100% mortality occurred by 24 hours. Both TRO and ORP decreased with time but, in all 25% treatments, cumulative mortality was greater at 48 hours than at 24 hours. This corresponded with post-exposure recovery data indicating that an initial exposure could result in delayed mortality. Mean LC50 values with confidence intervals could not be calculated for the 0-hour test. LC50 values calculated for 24- and 48-hour tests based on the percent of ozonated water were similar (30% and 26%); it is believed that the LC50 for the 0-hour test would be within 10% of this lower percentage. Mean LC50 values could not be calculated from ORP and TRO data at 0 hours but estimates fit in with results from 24 hours and 48 hours. This would again indicate the water is not losing toxicity over time.

These results support the hypotheses that bromine is an ozone-produced residual oxidant of toxicological importance and that bromine persists in ozonated waters (Sections 3.2 and 3.3). The practical result is that the bromine residual would likely be stable in ozonated ballast waters and would continue to have a biocidal effect if ozonated waters remained in ballast water tanks. However, loss of oxidants to the atmosphere in open containers did occur over the course of each 48-hour experiment and investigators will have to consider the implications of this in estimating overall toxicity. Furthermore, this suggests that ozone-produced oxidants may dissipate relatively quickly in systems that are exposed to the atmosphere, or perhaps from samples that are bubbled with ozone-free ambient air.

7.10.6 Oxidant/Tubing Study

TRO and ORP measurements of water samples that had been siphoned were not significantly different from TRO and ORP of water in the aquarium at any depth (Tables 7.10.6.1, 7.10.6.2 and 7.10.6.3). Therefore, clean, new plastic tubing should be acceptable as a means of collecting water samples without biasing oxidant measurements. However, it cannot be predicted whether results may be altered if this tubing has accumulated any organic or inorganic material on its inner walls.

Table 7.10.6.1. TRO and ORP values of ozonated water from the top third of the aquarium pre- (aquarium) and post-siphoning.			
Measurement	Top – Aquarium	Top – Siphoned	p-value
TRO (mg/L)			0.16
	7.75	7.15	
	7.6	n.a.	
	7.25	7.2	
ORP (mV)			0.19
	753	753	
	752	755	
	754	756	

Table 7.10.6.2. TRO and ORP values of ozonated water from the middle third of the aquarium pre- (aquarium) and post-siphoning.			
Measurement	Top – Aquarium	Top – Siphoned	p-value
TRO (mg/L)			0.51
	6.6	6.65	
	6.8	6.45	
	7.75	7.15	
ORP (mV)			0.61
	760	761	
	763	762	
	763	766	

Table 7.10.6.3. TRO and ORP values of ozonated water from the bottom third of the aquarium pre- (aquarium) and post-siphoning.			
Measurement	Top – Aquarium	Top – Siphoned	p-value
TRO (mg/L)			0.09
	4.6	5.65	
	4.65	8.55	
	4.55	7.3	
ORP (mV)			0.52
	768	768	
	769	769	
	769	771	

7.11 Summary of Shipboard Organism Removal Efficiencies vs. Ballast Water Exchange

The following figures summarize ozone system effectiveness (i.e., percent kill or "removal" of marine organisms) in comparison to two different benchmarks for BWE efficiency. The first, 95%, is a hypothetical expectation of nearly complete removal of coastal organisms that is considered theoretically obtainable during most BWE events. The second, 64%, represents the actual average exchange efficiency that was observed on board the *S/T Tonsina* for zooplankton during this study (Section 7.8).

When organism removal efficiency from ozone treatment was compared to the hypothetical target of 95% BWE efficiency, ozone would appear less effective than BWE in the removal of marine organisms. Only 7 out of 20 possible comparisons exceeded the 95% target, with bacteria in all experiments exceeding this target, and dinoflagellates, microflagellates, and sheepshead minnow removal efficiency exceeding this target only after 10 hours of ozonation in Experiment 3 (Figure 7.11.1). If both dead and moribund organisms are considered together, the success rate modestly improves to 11 out of 20 possible comparisons exceeding 95% removal efficiency (Figure. 7.11.2).

In contrast, organism removal efficiencies exceeded the actual mean removal efficiency observed on the *S/T Tonsina* of 64% in most cases. When only mortality is considered, ozone removal efficiencies exceeded that of actual BWE in 16 out of 20 possible comparisons (Figure 7.11.3). This improved to 17 out of 20 possible comparisons if both dead and moribund organisms are considered together (Figure 7.11.4). Assuming that the moribund organisms are likely to die 1 - 2 days after ozonation (Section 7.10.5), this is probably the most realistic comparison. Therefore, the empirical data suggest that ozonation is likely to be more effective than BWE for removal of many types of marine organisms from the ballast water of this particular vessel. It should be remembered, however, that this conclusion can not be extended to benthic organisms at this time. Benthic species were not sampled in BWE experiments, and they are likely more resistant to ozone than are pelagic species. Additional study is thus required to evaluate the efficacy of ozonation vs. BWE on the removal efficacy of benthic marine organisms.

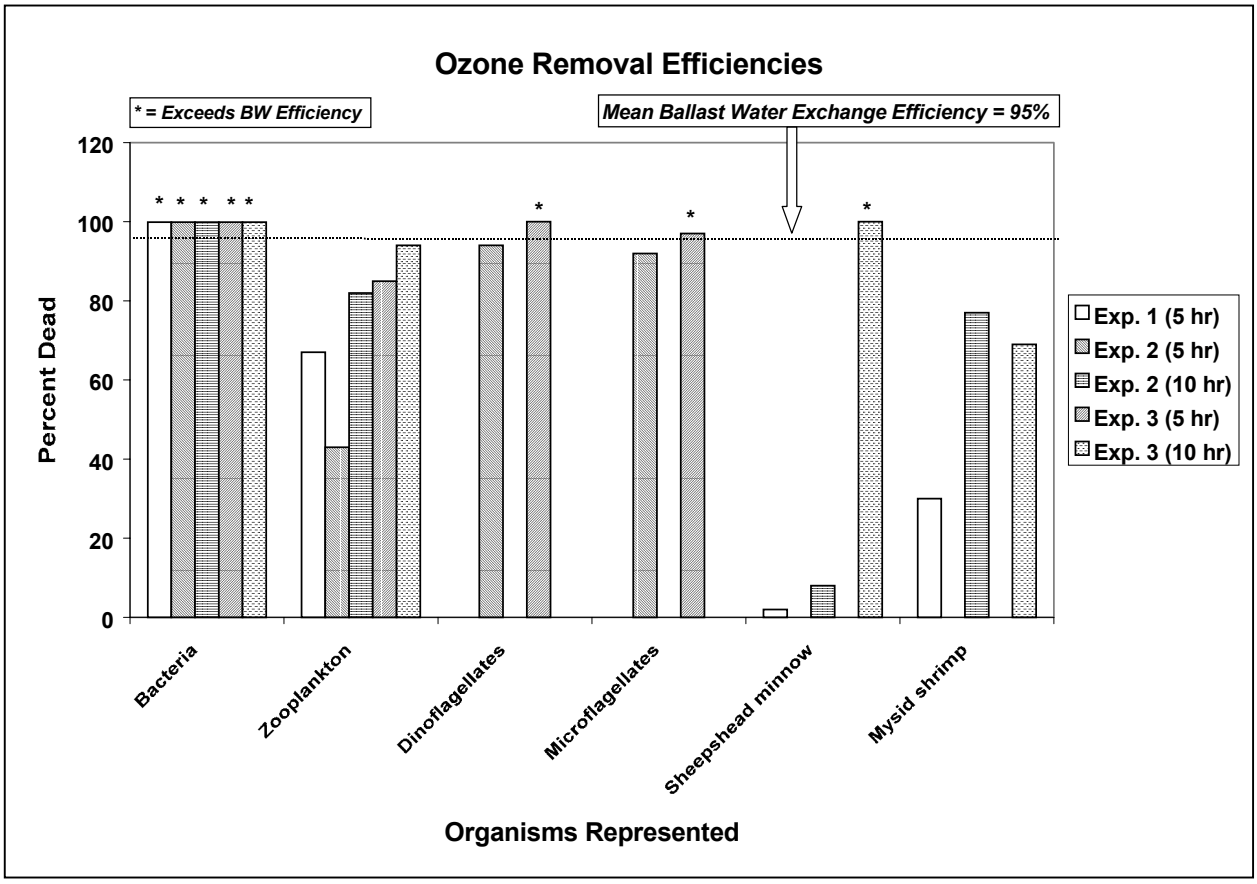


Figure 7.11.1: Percent mortality in ozone treatments compared to 95% hypothetical target for ballast water exchange organism removal efficiency.

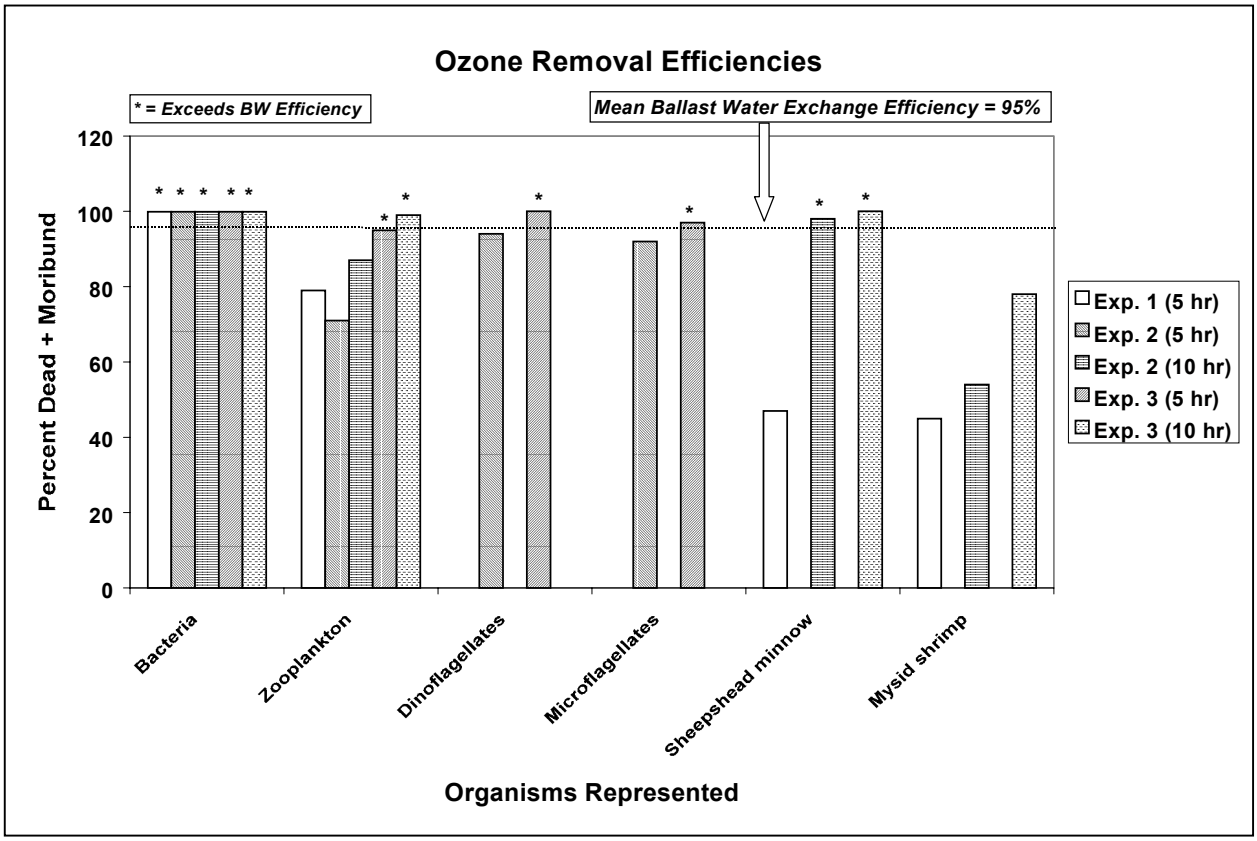


Figure 7.11.2: Percent dead + moribund organisms from ozone treatments relative to 95% hypothetical target for ballast water exchange organism removal efficiency.

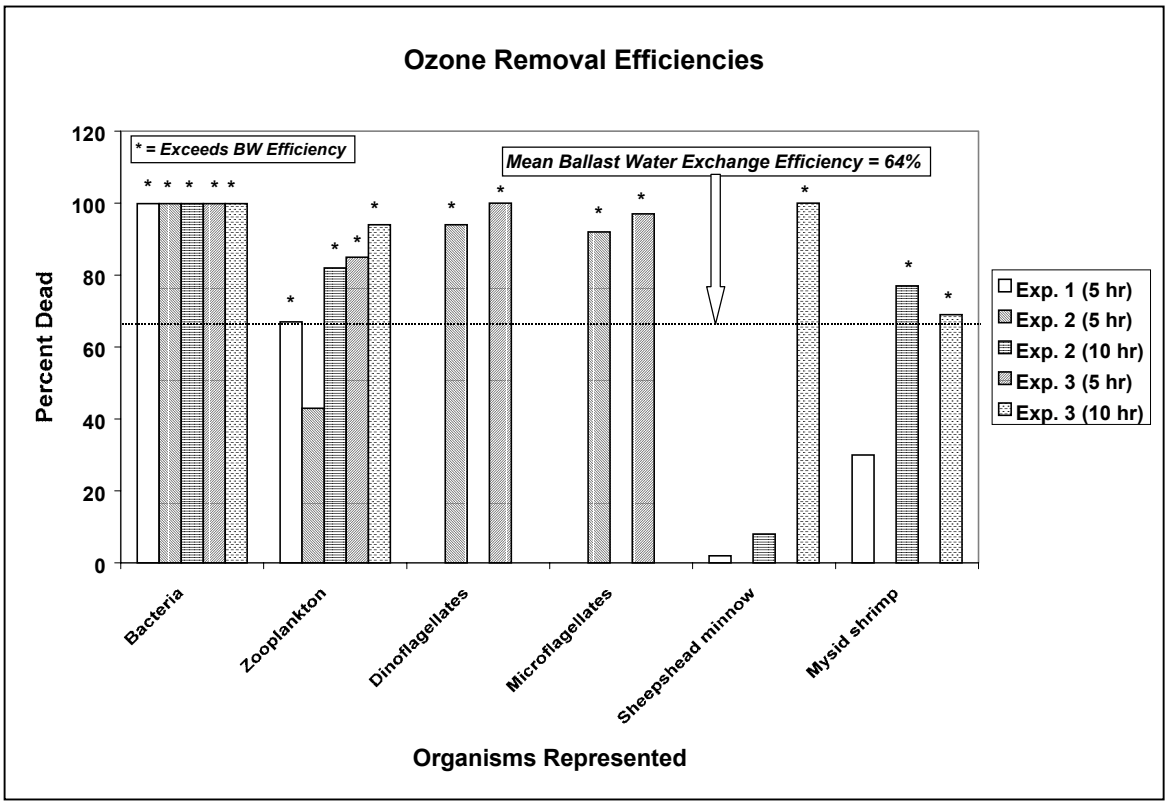


Figure 7.11.3: Percent mortality in ozone treatments vs. 64% mean organism removal efficiency from ballast water exchange on the *S/T Tonsina.*

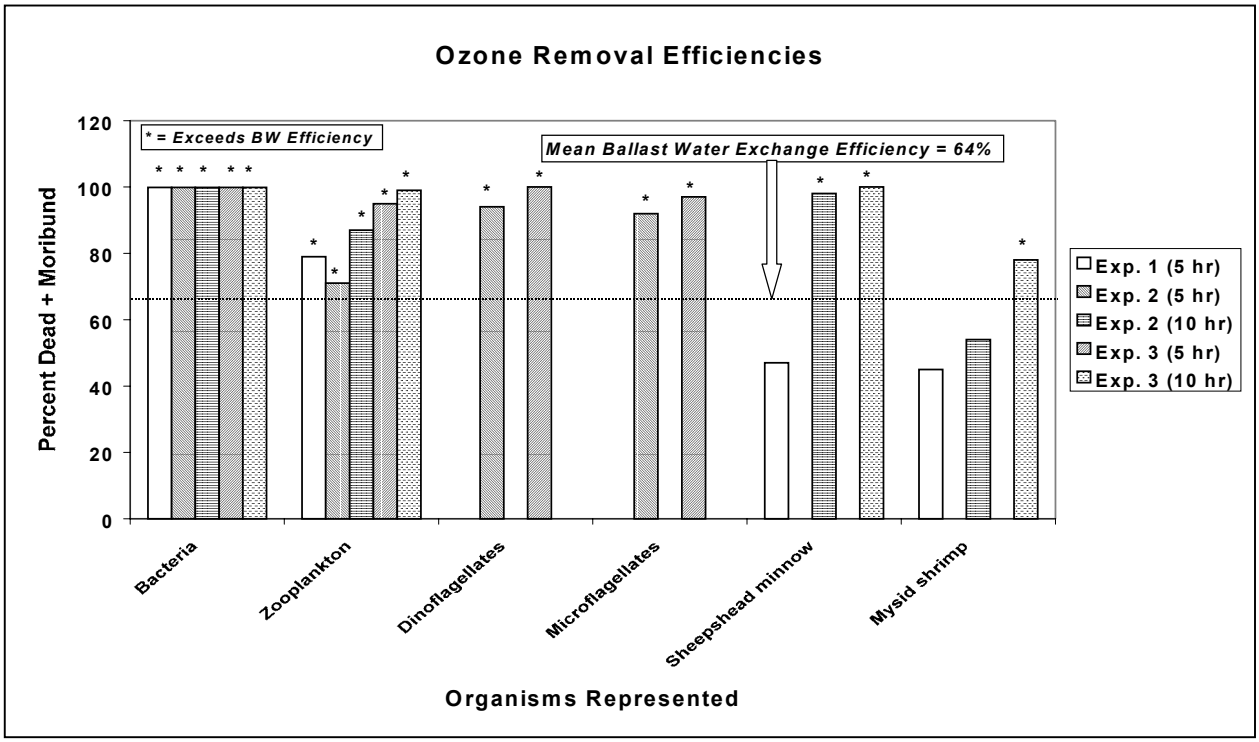


Figure 7.11.4: Percent dead + moribund in ozone treatments vs. 64% mean organism removal efficiency from ballast water exchange on the *S/T Tonsina.*

8 CONCLUSIONS AND RECOMMENDATIONS

The study described in this report represents the first of what is hoped will be several experimental phases designed to provide a full evaluation of the efficacy of the prototype Nutech O3, Inc. ozone system that is currently installed on the *S/T Tonsina*. The primary goal of this present study was to conduct a field-scale test of the operation and effectiveness of this ballast water treatment system for removal of a wide range of coastal marine organisms. While earlier studies suggested that the process was quite effective on bacteria, its performance with respect to higher organisms at the field scale was untested prior to the present study.

The specific objectives of the present study were to:

1) Determine the disinfection effectiveness of a full-scale ozone system in comparison with BWE efficiency.

2) Determine the acceptability of discharging treated ballast water using whole effluent toxicity testing, and to determine the latent toxicity of the subsequent ballast water discharge.

3) Obtain operational experience with the prototype ozone system in order to implement further system improvements.

8.1 Ballast Water Exchange

Two BWE experiments were conducted during this study. The organisms that were used to evaluate the efficacy of BWE were phytoplankton and zooplankton. Benthic organisms such as amphipods and shore crabs that were included in the ozone studies were not included in the exchange experiments. This was because ballast tanks have large structural elements that attenuate flow and thus provide refuge for these organisms. Furthermore, large organisms tend to be more rare in ballast tanks than small organisms, and those in benthic habitats are difficult to sample. Although it is believed that BWE will be less efficient at removing organisms from benthic habitats, this remains largely untested.

We have summarized data for zooplankton and some phytoplankton; however, additional phytoplankton analyses are in progress.

Conclusion 8.1.1: *The average efficiency of BWE on the S/T Tonsina was 64 % considerably less efficient than 95%, and may be less than that measured on other vessels.*

> **Recommendation 8.1.1.1.** The direct comparison of BWE and ozone treatment on the same vessel is critical in evaluating the ozone treatment effectiveness. Moreover, the test results (1) underscore the variation the can exist within ship type, and (2) suggest the level of "kill" needed for ozone treatment to surpass BWE aboard the *S/T Tonsina* may be lower than that for other vessels.

Recommendation 8.1.1.2. Additional exchange experiments should be performed aboard the Tonsina to improve statistical confidence in the estimate of BWE efficiency. Importantly, these experiments would extend the taxa and conditions under which exchange is measured, creating a more robust measure. These results should be compared formally against other exchange experiments for oil tankers (e.g., Ruiz, et al.), to test whether results from the Tonsina are statistically lower.

Recommendation 8.1.1.2. Future exchange experiments should include use of rhodamine dye tracer, to determine the amount of water actually replaced by exchange. The results from such an approach, although not necessarily a good proxy for removal of biota, can effectively test whether the physical removal of water is more or less efficient on the Tonsina compared to other ships.

Recommendation 8.1.1.3. It is critically important to alternate treatments (control, exchange, and ozone treatments) among tanks, to properly control for the effect of individual tank characteristics on mortality of target taxa. If the same treatment is always run in the same tank, it is not possible to separate effects of treatment and tank (as independent variables). [Note: this last recommendation applies to all treatment comparisons.]

8.2 Ozone Chemistry

For potential treatment chemicals, it is important to understand the stability of the chemical, the formation of reaction by-products that are transient and those that are stable, and their effects on receiving water when the treated ballast water is discharged. Three reaction by-products were identified from the literature as possible constituents of ozone treated ballast water. These were bromate ion, bromoform, and bromine (in water as hypobromous acid/hypobromite ion; $HOBr/OBr^-$).

Conclusion 8.2.1: *Ozone rapidly decomposes in marine ballast waters.*

In seawater where there is a significant concentration of bromide ion, ozone is catalytically destroyed with a half-life of five seconds. As expected there was no ozone observed in any of the ballast water samples that were analyzed. Therefore, ozone *per se* can be considered a good oxidant for the disinfection of marine ballast water because it is not chemically persistent.

Conclusion 8.2.2: *The only stable ozone-bromide ion reaction by-product that was detected was bromoform; however, from a review of the toxicological literature, the concentrations that were observed are not thought to have any adverse effect on the receiving waters.*

Bromate ion was never observed in the samples, suggesting that the lower pH of the coastal water favored the formation of HOBr that does not react with ozone to form bromate ion. In water treatment, bromoform results from the addition of chlorine to water containing bromide ion. The oxidation of the bromide ion by chlorine (hypochlorous acid/hypochlorite ion, $HOCl/OCl^-$) results in the formation of hypobromous acid/hypobromite ion ($HOBr/OBr^-$). The

reaction of this oxidized form of bromine with naturally occurring organic matter results in the formation of bromoform.

The appearance of bromoform and the fact that no bromate ions (or chloroform) were detected in any of the experiments indicates that $HOBr/OBr^-$ was formed during the ozonation process. The bromoform concentration increased with ozonation time. The increase is likely due to the continued formation of $HOBr/OBr^-$ with time, and to the reaction of the bromine with the natural organic matter present in the ballast water.

It is possible that additional bromoform formation could occur during the transit time during the return trip to the port of origin.

Recommendation 8.2.2.1: Although bromoform generation did not reach toxic levels in the present experiments, the possibility of additional bromoform formation during longer ozonation periods may need to be evaluated to ensure that toxic concentrations would not be reached.

Recommendation 8.2.2.2: As a part of future experiments, samples of the ballast water of ozonated tanks should be taken to detect bromoform, over an extended time period (e.g. during the return trip to the port of origin) to determine whether an increase in the bromoform concentration is observed.

Conclusion 8.2.3: *Seawater ozonation produces a chemical by-product, $HOBr/OBr^-$, that is relatively stable.*

The treated ballast water samples were shown to contain an oxidant as measured by the total residual oxidant test (TRO) using the iodide ion/DPD method for total oxidants. The presence of an oxidant, as a result of the oxidation of the bromide ion, was observed in all experiments. In Experiments 2 and 3, its concentration increased with time to greater than the range of the test procedure (4.5 mg/L as Cl_2) in the last two time periods sampled (7.5 and 10 hours after ozonation was begun). As stated above, the oxidant has to be $HOBr/OBr^-$.

The $HOBr/OBr^-$ are extremely labile in sunlight and would not persist long in the environment. However, it is possible that the bromine residual is stable or degrades slowly in the dark ballast tanks during the return voyage of the vessel. This would result in increased biocidal effect in the tanks as the vessel steams back to the port of origin. This is consistent with the WET tests where toxicity was observed after the samples were shipped to the testing laboratory. In laboratory tests, where samples of seawater were ozonated and in which toxic concentrations (as high as 12 mg/L measured as TRO) of bromine were generated by ozonation, the bromine as TRO was persistent over at least 2 days in closed containers.

Additional testing should be conducted to determine the stability of the $HOBr/OBr^-$ in the ballast tanks. It is highly likely that additional disinfection will be observed on the return voyage even after relatively short-term (e.g., less than 10 hours) ozonation periods. Samples of the biota

should be obtained to determine the extent of the additional disinfection during a longer time period than just during the ozonation.

Because HOBr/OBr^- are not gaseous and will mix with the ballast water, it may be that the formation of this oxidant will increase the overall effectiveness of the disinfection process through mixing in the ballast water tanks during the normal movements of the ship.

Recommendation 8.2.3.1: A series of sampling tubes should be installed in at least two ballast water tanks that are used as the treatment and control tanks, to enable sampling with time during a normal voyage of the *S/T Tonsina*. These tubes should be placed in areas that are close to sampling points that are accessed during the routine sampling, and in areas that are remote from the ozone diffusers. This will assist in an evaluation of the effect of mixing on the overall disinfection of the ballast water tanks on the voyage to the port of origin.

Recommendation 8.2.3.2: As a part of future experiments, determine the stability of the HOBr/OBr^- in the ballast water (in the dark).

Recommendation 8.2.3.3: Sample the ballast water tanks for selected biota, during the return trip to the port of origin, to determine the extent of disinfection that occurs with time in the waters as it may increase beyond the time frame of current measurement.

Recommendation 8.2.3.4: The measurement of bromine as TRO using a colorimetric field test procedure is similar to measuring chlorine in water and wastewater treatment plants. Therefore, it is possible that TRO could be used as a control measurement for the ozone process in marine ballast water systems. Additional studies should examine this measurement as a process control technique.

Conclusion 8.2.4: *Seawater ozonation produced HOBr or OBr^- that can be toxic to marine organisms up to 48-hours after ozonation.*

As part of the regulatory process for the approval of a ballast water chemical treatment process, the treated water must be screened using standard whole effluent toxicity (WET) tests. During this experiment, WET tests were conducted on ozone-treated and untreated ballast water from three field trials.

Results of these tests using treated ballast water from the experiments conducted on the *S/T Tonsina*, with the mysid *A. bahia* and the topsmelt *A. affinis*, indicated that ozonation byproducts were stable enough to cause toxicity (30-80% ozonated ballast water causing acute mortality) in ballast waters even 1-2 days after ozonation. However, no chemical measurements were conducted in these tests to quantify ozone-produced oxidants.

Therefore, mysids were exposed to ozone in the laboratory using experiments of similar design to the WET tests. Tests were initiated with ozonated waters that were 0-, 24-, or 48 hours-old and measured ORP and TRO at all significant time points. As implied by the WET tests, oxidants did not disappear from ozonated waters held 24 or 48 hours in a sealed container

at 12 ºC. All organisms died when exposed to 50, 75, or 100% ozonated water in tests initiated at any of the three time points (0 hours, 24 hours, 48 hours). The ORP was greater than 720 mV and TRO greater than 1.76 mg/L in treatments where 100% mortality occurred by 24 hours.

The presence of bromine in the ballast water could provide additional disinfection in the ballast water tanks during a normal return voyage to the port of origin. In many areas throughout the U.S., standard wastewater treatment operations include chlorination and de-chlorination, prior to discharge of the wastewater into the receiving waters. A common chemical that is used for this is sodium bi-sulfite, $NaHSO_3$. The reduction of the oxidant is almost instantaneous and eliminates the adverse environmental effects of the oxidant (chlorine) in the receiving waters.

These results support the hypothesis that ozone-produced oxidant, $HOBr/OBr^-$, can persist in ozonated waters, and can be lethal up to 48 hours after ozonation. Seawater ozonation thus produces relatively stable by-products whose toxicity may need to be considered or treated to ensure safe discharge of ozonated ballast water.

> **Recommendation 8.2.4.1**: Parallel WET tests should be conducted on treated ballast water with and without the addition of a reductant, such as $NaHSO_3$. These tests would provide data necessary to determine the extent to which the effluent toxicity that has been observed was due to the presence of bromine. These tests would also allow for the design of an entire treatment system for seawater ballasting in ships.

> **Recommendation 8.2.4.2**: The observation that bromine was present in the ballast water suggests that disinfection was a combination of ozone and bromine. It also suggests that the present study underestimated the disinfection efficiency of the ozone treatment because samples were only obtained immediately after the ozonation was stopped. The effect of the stable reaction by-product, HOBr/OBr-, should be studied for its additional disinfection over longer periods of time (during the return voyage to the port of origin).

Conclusion 8.2.5: O*xidation-reduction potential, ORP, increased with time of ozonation, reaching a plateau of about 700-800 mV.*

The ORP of the sample solutions increased with ozonation time, although they appeared to increase rapidly from 0 to 2.5 hours and then increase more gradually to a plateau of ca. 800 mV. This ORP "plateau" was observed in both laboratory and field studies, and coincided with significant mortality in most of the species tested. It appears that ORP may be a good measure of the effectiveness of ozone in ballast water treatment, with levels from ca. 700-800 mV corresponding to significant mortality in most cases. This is also consistent with the commonly used target of 700 mV ORP as the basis for ensuring and controlling marine aquarium exhibit disinfection.

> **Recommendation 8.2.5.1:** Additional experiments should be conducted in the laboratory and on the ship to determine the potential of ORP as a control measure for the ozone treatment process.

8.3 Effects of Ozone on Bacteria

Conclusion 8.3.1: *The ozone process was extremely effective at eliminating culturable bacteria from the ballast water.*

The viability of heterotrophic bacteria in treated and untreated ballast water was determined using a complex marine bacteriological medium, called Marine R2A. Samples collected on board the *Tonsina* were transported to the University of Washington laboratory for processing. To increase the sensitivity of the protocol for samples collected from ozonated ballast water, water samples were passed through a membrane filter and the concentrated sample was placed on the agar medium. For the treated ballast water, the pretreatment samples contained between 10^5 and 10^6 colony forming units per liter. After 10 hours of treatment, the population level was reduced to less than 3 to 5 colony forming units per liter, which is a reduction of greater than 99.99%.

Recommendation 8.3.1.1: Future studies should use both culturable and direct counting methods to estimate the total number of microorganisms and the percentage inactivated by the ozone process.

Recommendation 8.3.1.2: Alternative microbial viability assays should be considered. Several investigators have suggested that measuring ATP (adenosine triphosphate) is an excellent indicator for the viability of microorganisms and other organisms since this compound rapidly degrades following cell death.

Conclusion 8.3.2: *Bacterial re-growth was not observed after 30 days storage in the dark in the laboratory.*

The viability of heterotrophic bacteria in treated and untreated ballast water that was held for a period up to 35 days was determined in the first and third September 2001 experiments. Control (untreated) ballast water had a culturable population of 10^6 colony forming units following 2, 7, and 35 days of storage. The treated ballast water had a population level that was below the detection limit, 3 colony forming units per liter, following 2, 7, and 35 days of storage. Therefore, ozone is effective in preventing the growth of bacteria for up to one month following a 10-hour exposure period.

Recommendation 8.3.2.1: Future studies should examine the toxicity of treated ballast water on microorganisms present in receiving waters. Laboratory experiments should be conducted with ozonated ballast that has been ozonated and stored for different lengths of time. The treated ballast water can be mixed with seawater or defined microbial cultures. The viability of organisms within the mixed water can be determined.

8.4 Effects of Ozone on Phytoplankton

Conclusion 8.4.1: *The ozone process was highly effective in reducing concentrations of dinoflagellates and microflagellates in the water samples. Although this likely results directly from mortality, some cyst formation in the bottom sediments may occur.*

> **Recommendation 8.4.1.1**. Future tests should measure the extent to which declines of phytoplankton in the water column are accompanied by cyst formation in the bottom sediments.

Conclusion 8.4.2. *The present analysis does not measure the effects of ozone treatment on diatoms because counting methods could not distinguish between living and dead organisms.*

> **Recommendation 8.4.2.1**. Future tests should use vital stains to distinguish viable from dead diatoms associated with treatment.

Conclusion 8.4.3: *Although exchange and ozone treatments appeared to reduce certain taxa of coastal planktonic organisms, the results were sometimes inconclusive for benthic organisms.*

8.5 Effects of Ozone on Larger Invertebrate and Vertebrate Animals

Conclusion 8.5.1: *The ozone process was not as effective against larger organisms that were introduced into the tanks (in cages) during these experiments, but which could find their way into ballast tanks under real world conditions.*

No shore crabs succumbed to the treatment system in any of the experiments; however, they were moribund at the 10-hour time point in Experiment 3. Over 90% of the amphipods survived in every experiment. Mysid shrimp approached effective elimination only during Experiment 3, while sheepshead minnows showed the greatest sensitivity of all the caged organisms with 100% kill in Experiment 3. These results were consistent with laboratory tests in which sheepshead minnows were the most sensitive to ozone exposure, followed by mysid shrimp, and amphipods were the least sensitive.

These results suggest a general trend of larger organisms being more resistant to ozonation than smaller organisms. From these data, however, it appears that there would be significant variation within this overall trend (e.g., sheepshead minnows being the most sensitive in caged and laboratory studies), and an insufficient number of different species were examined to establish this theory with any statistical rigor. Also, physiology, behavior, or both may reduce toxicity to even small organisms (e.g. amphipods).

> **Recommendation 8.5.1.1:** The effect of residual toxicity on the population dynamics of residual organisms needs to be studied to quantify the ecological significance of morbidity vs. mortality in short-term experiments.

Conclusion 8.5.2: *Latent toxicity may enhance the overall performance of ozone treatment.*

In the caged organism studies, performance levels increased in several categories when not only dead but also moribund organisms were considered "eliminated from the system." It is quite possible that moribund organisms, which were removed from the ozonated water and their vitality judged immediately following the treatment, will have lost their reproductive capabilities or even succumb to the effects of the treatment within days or hours following the treatment. During normal vessel operating conditions, all organisms will remain in the ozonated water for up to 3.5 days following the treatment and some level of latent toxicity similar to those observed in the WET testing would result in increased treatment efficiency.

The sampling and experimental methods used in this study to evaluate the viability of benthic organisms (crabs and amphipods) were probably more conservative than necessary by ignoring latent toxicity or delayed death. While organisms "surviving" a BWE are likely to remain highly viable, potentially enabling them to proliferate as an invasive species, the same can probably not be said, or at least not to the same degree, for organisms surviving an ozone treatment in which a large fraction of the population is killed. In the latter case, the "surviving organisms" are likely damaged/impaired to some degree and may never recover sufficiently to reproduce. The post-exposure toxicity studies were consistent with this assertion. If this proves to be the case, the organisms are no longer an invasive species threat and should be considered "dead" in the context of the treatment process. It is clear that additional population-level studies are required to answer this question.

> **Recommendation 8.5.2.1:** That in future studies samples be obtained from the ballast tanks that have been treated with ozone, over the course of days, to assess the effect of the residual oxidants on the viability of organisms.

8.6 Ballast Water Exchange vs. Ozone Treatment

Conclusion 8.6.1: *While exchange and ozone treatments appeared to reduce certain taxa of coastal planktonic organisms more effectively than did BWE on the same vessel, the results may be inconclusive because of the confounding interactions of tank, time, and habitat of the organisms.*

> **Recommendation 8.6.1.1:** There are several ways that future ballast treatment/exchange experiments could be strengthened. First, the location in which ballast water was taken could be changed to an area that has less interaction with ocean water. Port Angeles Harbor is located on the Strait of Juan de Fuca, which is directly connected with and influenced by the Pacific Ocean. Ballast water taken on, in inland waters such as Puget Sound or an embayment such as San Francisco Bay would have a much more discrete coastal planktonic fauna. Second, multiple replicates of treatment and control tanks would add needed statistical power to analyses. Third, treatment should be alternated among tanks, to remove the effects of individual tanks *vs*. treatments.

8.7 Laboratory toxicity Studies

Conclusion 8.7.1: *The toxicity of ozonated seawater correlated with both ORP and TRO levels, with toxicity thresholds being broadly similar to those observed in the caged studies.*

Median lethal concentrations (i.e., LC50) for all but one species exposed to ozonated artificial seawater in the laboratory ranged from 698 - 768 mV ORP, and from 1.29 - 2.93 mg/L TRO. 50% mortality was never achieved for the amphipod, *Leptocheirus plumulosus*. These data were consistent with results from the caged organism studies in which mortality (at least for mysids) also was strongly correlated to ORP measurements. Therefore, ORP measurements ranging from 700-800 mV appear to be associated with significant acute mortality in a variety of marine species both in the field and in the laboratory. TRO would appear to be toxic at levels ranging from 1-3 mg/L, but literature studies suggest that even lower concentrations (e.g., < 1 mg/L) may also be acutely lethal. Unfortunately, the TRO dose-response curves were less consistent than ORP curves, so there is more uncertainty in quantifying effective ozonation thresholds as a function of TRO.

Furthermore, the relative sensitivity of test species exposed to ozone (as measured by ORP) was similar in both the field and lab experiments, with only limited exceptions. In the caged studies, the sheepshead minnow *C. variegatus* was the most sensitive species, followed by mysids (*A. bahia*) and amphipods (*R. abronius*). In the laboratory, LC50 values for *C. variegatus* were indeed lower than *A. bahia*, suggesting that the sheepshead minnow was slightly more sensitive with respect to ORP exposure. One of amphipod species tested in the laboratory (*L. plumulosus*) was less sensitive to ORP than either sheepshead or mysids, but the second (*R. abronius*) exhibited similar sensitivity to sheepshead minnows.

> **Recommendation 8.7.1.1**: ORP readings may provide a means of quantifying effective ozone exposures in seawater, with significant mortality likely to occur when readings approach 700-800 mV. TRO may also provide an effective means of quantifying effective ozone dose, but the specific threshold may be somewhat less consistent than ORP. Perhaps both measures can be used in combination to help control the timing and effectiveness of ballast water ozonation procedures. However, some level of species-sensitivity to ozone will need to be considered in the design or operation of an effective treatment system.

Conclusion 8.7.2: *It may be that only relatively short-term ozonation is required to produce acutely lethal concentrations of ozone by-products in seawater.*

Limited mysid mortality (30-60%) occurred within the 1.5 hours of ozone exposure in laboratory experiments where TRO concentrations exceeded 4.0 mg/L. However, 100% mortality was observed in those survivors 48 hours after transfer to clean seawater. No mortality was observed within 1.5 hours or at 24 hours post-exposure when TRO measurements were less than 1.0 mg/L, but 60% mortality occurred by 48 hours post-exposure. Therefore, it appears that

sufficient amounts of bromine oxidants built up in the ozonated water over 1.5 hours to have induced both immediate and, to an even greater extent, delayed mortality (up to 48 hours later).

> **Recommendation 8.7.2.1**: Studies are needed to confirm whether relatively short ozonation periods are all that is necessary to provide effective kills, so long as sufficient amounts of relatively stable by-products (e.g., 1 - 4 mg/L TRO) are produced. Because the bromine residual is likely to be stable in ozonated ballast waters (in the dark), it could continue to have a biocidal effect over at least 1-2 days. However, loss of oxidants to the atmosphere in open containers did occur in laboratory experiments, suggesting that ozone-produced oxidants may dissipate relatively quickly in systems that are exposed to the atmosphere, or perhaps from samples that are bubbled with ozone-free ambient air. Thus, studies should be designed to quantify the timing and effects of residual (i.e, bromine) toxicity as a function of the fate and distribution of ozone-produced oxidants in open vs. closed systems.

8.8 Engineering Considerations

Conclusion 8.8.1: *The level of disinfection varied with location and possibly depth within the ballast water tank.*

Marked variation in the effectiveness of the ozone treatment system to treat (kill or inactivate) phytoplankton, zooplankton and caged organisms was dependent on the sampling location's relative proximity to the diffusers. In the forward column A of the treatment tank, 91 % of the zooplankton were dead after five hours of ozonation in Experiment 1. But in the aft column B of the same tank, only 47 % of the zooplankton died. In Experiment 2 the differences were reversed: after 10 hours of ozonation, 97 % of the zooplankton were dead in column B of the treatment tank, but only 67% were dead in column A. In Experiment 3, where ozone delivery was maximized in the vertical wing tank (which was the tank being sampled), the difference between the forward and aft columns decreased to less than 5 %.

The results of the caged organism study reflect variation by location (sampling port A or B). Mysid shrimp best illustrate this variation by displaying an intermediate resistance to ozone compared to the relatively susceptible sheepshead minnows and relatively hardy amphipods and crabs. In Experiment 1, 20% of the mysid shrimp deployed 10 feet from the bottom of the tank in column A survived, while 60% of those deployed 50 feet from the bottom survived. In contrast, 100% of all mysids survived at all depths in column B. In Experiment 2, 90% survived at the bottom depth in both columns, but none of the mysids at the top depth survived in column A while 78% survivorship was observed in column B. In Experiment 3, 100% survived at the top depth in column A but none survived at the same depth in column B, while at the bottom depth the numbers were reversed: 100% survival in column A and 0% in column B.

This variation in effect within the same tank suggests that the ozone was unevenly distributed throughout the tank. This could be because the extent of ozone mixing within the tank, both vertically and horizontally, was less than expected. Only 16 diffusers, placed at or near the bottom of the wing tank, were designed to treat the entire side tank. This differs from

the double bottom ballast tank areas located underneath the cargo holds, which have multiple rows of diffusers that treat a much shorter water column.

Recommendation 8.8.1.1: The ozone distribution system requires re-engineering to improve ozone delivery and mixing. Specifically, further research is warranted to determine either more optimal locations for the ozone diffusers, or to provide means to insure better circulation within the tank to better use the ozone and its byproducts. As part of the prototype system installed for these tests, a "purge air" system was installed. This system utilizes higher volume compressed air at a significantly higher pressure than can be developed by the ozone injection system, and so it could possibly be used to improve the circulation in the tank, once the ozone treatment has been completed. The purge air system is connected through valves to the same distribution piping and diffusers used by the SCX 2000.

8.9 Safety Considerations

Conclusion 8.8.1: *The presence of pressurized, oxygen-enriched air/ozone gas in proximity to hydrocarbon liquids and vapors as will exist on the S/T Tonsina during routine operations of the ozone system can create unsafe conditions if combustible or explosive mixtures are formed.*

The prototype system did develop ozone leaks at several locations and instances over the test period. At times, the odor of ozone was noticeable at certain locations on deck and in a control room.

Recommendation 8.8.1.1: These potential health and safety risks, their impact on system design, material selection, engineering controls, and O&M (Operation and Maintenance) procedures and demands, should receive careful consideration in the overall ozone decision process.

Recommendation 8.8.1.2: Consider single-point injection of ozone into the water intake stream in lieu of the current distribution system. However, this will require even more ozone capacity because the ballast water intake time is much shorter than the typical 3.5-day voyage time available for in-situ ozonation.

8.10 Other Considerations

Conclusion 8.10.1: *Use of sediment-suspending flocculent in ballast water appears to have little impact on the effectiveness of ozonation, but data are few.*

Sediment in ballast water poses a complication to any effort to kill biota in ballast tanks. As the sediment accumulates in the corners and interstices of a ballast tank, it can provide habitat for benthic organisms as well as a potential refuge from exposure to whatever ballast water treatment that is being employed. To be completely effective, ballast water treatment technologies must therefore treat not only the biota present in the water column, but also biota resident in the accumulated sediment within the tank.

As a regular part of their ballasting operation, the *S/T Tonsina* crew injects a flocculent (Mud-Out®) into the incoming ballast water that tends to suspend sediment in the water column and thus prevent it from accumulating in appreciable quantities or densities in the ballast tanks. The purpose of this operation is to reduce the workload of the crew, who would otherwise have to manually remove the sediment from time to time as it built up in the tank.

Preliminary tests that were conducted suggested that Mud-Out® has very little effect on the effectiveness of ozonation. In laboratory tests with artificial seawater, Mud-Out® additions had no significant impact on ORP levels, but after 3 hours of ozonation, caused a very minor decrease in TRO concentrations. It is thus unlikely that Mud-Out® represents a significant chemical ozone demand that would impair the effectiveness of the ozonation system.

> **Recommendation 8.10.1.1:** Even though Mud-Out® does not appear to negatively impact the effectiveness of ozonation from a chemical point of view, it might be desirable to confirm this biologically. Furthermore, the ultimate impacts of sediment anti-flocculants on the possible transfer of non-indigenous species are warranted.

Conclusion 8.10.2: *The ozonated ballast water was supersaturated with oxygen.*

Although this study targeted the effects of ozone, it should be mentioned that the dissolved oxygen levels in the ballast water increased significantly during the ozone treatments. This is of importance in considering guidelines for obtaining a discharge permit for the treated ballast water. Two oxygen sources contributed to this increase: 1) the dissociation of ozone contributes 0.67 mg O_2 for every one mg O_3; and 2) the applied "ozone gas" is actually a mixture of ozone, oxygen, and small amounts of nitrogen. Oxygen in the "ozone gas" was a partial contributor to dissolved oxygen levels. In Experiment 2, ozone dissociation could have contributed up to 5.8 mg/L oxygen to the ballast water through complete dissolution and dissociation. Yet, the dissolved oxygen concentration increased 8.6 mg/L on average by the end of the treatment. (The controls increased by 1.7 mg/L, the exact reason for this increase is not known). Elevated oxygen levels can affect the viability of organisms but no attempt was made in this study to distinguish between the effect of ozone and oxygen, as oxygen was an integral part of the overall treatment.

> **Recommendation 8.10.2.1**: The high oxygen concentration in the ballast water is a side benefit of the process. Ballast water that is saturated (or supersaturated) with oxygen prior to being discharged into the receiving water will have an insignificant biochemical oxygen demand. Ballast water with no biochemical oxygen demand will be acceptable for discharge. Therefore, that portion of the permitting process will not create any concern on the part of regulators.

9 FUTURE RESEARCH DIRECTIONS

To address the recommendations that have resulted from this Phase I study, the following is an outline recommending a number of experiments and considerations for future research.

1. The finding that bromine (hypobromous acid/hypobromite ion) is present in the ozonated ballast water, and that the concentration increases during the treatment, suggests that the concentration of bromine should be more reliably determined (many samples exceeded the detection limit of the test procedure). This would allow for verification of bromine concentrations on board ship, and a determination of the stability of bromine residuals in the ballast tanks.

   Accurate bromine concentrations should be determined at several times after ozonation, during a normal voyage returning to Valdez. It should also be determined immediately prior to dumping the ballast water.

2. If bromine is found to be relatively stable over a period of time in the ballast water, it is quite possible that the exposure of organisms to this oxidant for an extended period of time would increase the overall effectiveness of the process. In particular, this may improve the effectiveness of the process with respect to larger or more sessile (e.g., epibenthic) organisms.

   Samples for the viability of different organisms should be obtained over the period of time that the vessel is in transit to Valdez. These data should be compared to bromine concentrations from the same samples to help evaluate residual effect concentrations.

3. If bromine is found to be relatively stable over a period of time in the ballast water, this could account for the toxicity observed in the Whole Effluent Testing (WET).

   Equivalent concentrations of bromine should be added to control samples for analysis in the same manner as those for WET testing. This will help validate the hypothesis that bromine is the ozone-produced residual that is responsible for latent toxicity. Quantifying this residual toxicity is the key to understanding the potential for bromine residuals to enhance the effectiveness of the ozonation system and the potential environmental risks of discharging ballast water that still contains bromine residuals.

4. If bromine is found to be stable in the ballast tanks, it is possible that additional bromoform also could be formed during the return trip to Valdez.

   Samples should also be taken for bromoform analysis to determine if further formation of bromoform is observed during the return trip.

5. The *BWE* experiments underscore the differences between different vessels. It is possible that these differences result from the amount of complex internal structure in the ballast tanks on the *S/T Tonsina*, compared to the other tankers' ballast tanks that have been examined.

   Exchange experiments, including the use of dye tracer and alternating tanks, should be replicated over time and space. Additional data will improve statistical confidence in the data and allow more thorough understanding of exchange as a

standard for comparison of ozone treatment performance. Further, it will be important to compare these results to exchange measures for the other oil tankers (n = 8) for which parallel data already exist.

6. At the end of the experiments, the dissolved oxygen concentration was near or exceeded saturation. The increased oxygen concentration in the ballast water will have a positive effect on lowering, if not eliminating, the biochemical oxygen demand (BOD) of the ballast water. BOD is one of the critical parameters for obtaining a NPDES permit.

   BOD should thus be determined for samples of ballast water prior to and after treatment, at the port of treatment and at the port of origin (Valdez) where the ballast water will be discharged.

7. With the finding that TRO, a bromine residual, is present, it may be possible to use a simple colorimetric test of TRO (that is total oxidants) for process control. This would be similar to the use of chlorine residual in drinking water treatment. However, ORP measurements may also help indicate when ozonation is reaching effective thresholds, and may perhaps be used in combination with TRO. An additional advantage is that ORP probes can potentially be used to engineer automatic ozone control systems.

   Studies should be designed to test the use of TRO vs. ORP as chemical parameters to facilitate control and, hence, maximize the effectiveness of the ozonation process.

8. With the discovery of an apparently stable oxidant, $HOBr/OBr^-$, in the treated ballast water, it is possible that during the trip back to Valdez, through normal mixing and diffusion, pockets that would have otherwise been untreated could be exposed to an oxidant residual for a period of up to two days. This would facilitate additional treatment in those locations improving the overall effectiveness of the process.

   Sampling tubes should be installed in the treatment and control ballast tanks in areas that are sampled by vertical Niskin casts, and also in locations that ozone may not reach to determine whether the additional $HOBr/OBr^-$ contact time would increase disinfection.

10. Microbial culture methods only enumerate microorganisms that are capable of growing on the selected medium under the chosen incubation conditions. In the present study, Marine R2A agar was used and the number of colony forming units per liter (CFU/L) was determined. No single medium or collection of media are capable of supporting the growth of all of the variety of organisms present in an environmental sample, but such studies can provide information about the viability of microbial populations. "In response to environmental conditions, bacteria may be present in a viable but nonculturable state by classical microbiological methods" (Gregori et al. 2001). Flow cytometry is an enumeration technique that increases the precision of total bacterial counts compared to CFU/L, and is a faster analysis that has less count bias than epifluorescence microscopy. If ozonation causes microbial cells to lyse, as it appears to do, then a direct enumeration procedure such as flow cytometry, could be utilized to measure the number of microorganisms present in treated and untreated ballast water.

Future studies should include flow cytometry development and testing using a FACScan cytometer or the best available instrument with a fluorescent nucleic acid dye for additional enumerations. This assay could be used in conjunction with previously described cultural sampling methods for bacterial counts.

11. An alternative method for determining the viability of living cells is to measure the quantity of adenosine triphosphate (ATP) in collected environmental samples. ATP is associated with living biomass and rapidly degrades following death of the animal or microbial cell. ATP measurements have been used for many years in aquatic microbial ecology.

    ATP measurements should be taken for treated and untreated ballast water samples. Although the ATP protocol has largely been used and developed for microbiological research, some preliminary experiments and development should be performed to use the assay for phytoplankton and zooplankton. Ballast water samples could be screened through a series of filters of different pore sizes so the different biological taxa could be separated by size. The ATP analysis could then be performed on the different sets of filtered material.

12. It is evident from analysis that spatial variation within a tank can be extensive, both with respect to distribution of biota and ozone. Furthermore, individual tanks may not be true replicate units in both respects, obscuring the effects of tank versus treatment.

    Sampling should occur at additional locations, beyond those used in this study, to include the full spectrum of conditions available within ballast tanks. In addition, the experimental treatments should be alternated among tanks, to control for the effect of individual tank on treatment effects.

13. Limited bacterial regrowth studies indicated that no regrowth occurred over 30 days.

    Additional studies are required to verify that no regrowth of microorganisms occurs in ozonated water.

14. No studies were conducted to examine the potential regrowth of phytoplankton or zooplankton.

    Studies should be conducted to better define the potential for regrowth of the phytoplankton and zooplankton that are found in the coastal ballast water.

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11 APPENDICES

11.1 APPENDIX A: Personnel assignments, sample volumes, and materials required for Niskin sampling

ORDER COLLECTED	TEST	VOLUME	REPS	TOTAL VOL	METHODS	PERSON	MATERIALS REQUIRED
1	OZONE	50 ML	2	150 ML	ACCU-VAC	1,2,3	(1) 250 ML BEAKER, 2 ACCU-VAC INDIGO
2	BROMINE	50 ML	2	150 ML	ACCU-VAC	1,2,3	(1) 250 ML BEAKER, 2 ACCU-VAC DPD'S
3	ORP, **DO, TEMP, PH, SALINITY**	250 ML	1	250 ML	PROBES/ DREL	1,2,3	250 ML WIDE MOUTH BOTTLE, DREL, PROBES, (5 INITIAL/1 OTHERS) 30
4	BROMATE	125 ML	1	125 ML	STORE ON ICE	1,2	(1) WIDE MOUTH AMBER BOTTLES, 125 ML
5	BROMOFORM	40 ML	2	80 ML	STORE ON ICE	1,2	(2) 40 ML AMBER GLASS BOTTLES
6	BACTERIA	1000 ML	3	3000 ML	STORE ON ICE	1,2	(3) 1 L NALGENE WIDE MOUTH BOTTLES, COOLER AND ICE
7	PHYTOPLANKTON	1000 ML	1	1000 ML	STORE (ON ICE?)	1,2	(1) 1 L NALGENE BROWN WIDE MOUTH BOTTLE PLUS FORMALIN
Initial sample only:							
8	**NITRATE**	25 ML	1	25 ML	FREEZE (DRY ICE	1,2	COMBINED (1) 250 ML WIDE MOUTH BOTTLE, DRY ICE
9	**NITRITE**	10 ML	1	10 ML	FREEZE (DRY ICE	1,2	COMBINED (1) 250 ML WIDE MOUTH BOTTLE, DRY ICE
10	**REACTIVE P.**	10 ML	1	10 ML	FREEZE (DRY ICE	1,2	COMBINED (1) 250 ML WIDE MOUTH BOTTLE, DRY ICE

* BOLDED TESTS ONLY DONE AT INITIAL TIME POINT FOR EACH TANK

11.2 APPENDIX B: Conceptual Framework for Testing Ballast Water Treatment (Ruiz et al. 2002)

11.2.1 Background

The worldwide transfer and introduction of nonindigenous species (NIS) by human activities is having significant and unwanted ecological, economic, and human-health impacts (e.g., OTA 1993, Wilcove et al. 1997, Pimentel et al. 2000). Although most attention to date has focused on invasions in terrestrial and freshwater habitats, it is evident that NIS invasions have become a potent force of change in coastal marine ecosystems. Roughly 400 marine and estuarine NIS are known to be established in North America alone, and over 200 of these species can occur in a single estuary (Cohen and Carlton 1995, Ruiz et al. 1997, 2000a). Some of these species have become numerically or functionally dominant in invaded communities, where they have significant impacts on population, community, and ecosystem-level processes (e.g., Cloern 1996, Crooks 1999, Ruiz et al. 1999, Grosholz et al. 2000).

Although many transfer mechanisms (or vectors) have contributed historically to the invasion of coastal habitats by NIS, shipping has been the vector responsible for many of the known invasions (Carlton 1979, Carlton and Geller 1993, Cohen and Carlton 1996, Hewitt et al. 1999, Ruiz et al. 2000a). Furthermore, the global movement of ballast water now appears to be the single largest transfer mechanism for marine NIS. Since the 19th century, ships have used ballast water for stability, discharging water both at ports of call and en route (Carlton 1985). Ports can receive relatively large volumes of ballast water, originating from source regions throughout the world. For example, the United States and Australia each receive annually >79 million metric tons of ballast water on ships arriving from foreign ports (Kerr 1994, Carlton et al. 1995). A taxonomically diverse community of organisms is entrained and transported within ballast tanks (e.g., Carlton and Geller 1993, Smith et al. 1999, Hines and Ruiz 2000, Ruiz et al. 2000b), resulting in many successful invasions of nonindigenous species at ports throughout the world.

BWE, or mid-ocean exchange, is currently the only management strategy available for ships to reduce the quantities of non-indigenous coastal plankton in ballast water (National Research Council 1996). Ships practice two types of BWE that replaces coastal water with oceanic water, reducing the initial concentration of coastal organisms (i.e., those that are most likely to invade a port). Flow-Through Exchange occurs when water from the open ocean is pumped continuously through a ballast tank to flush out coastal water, and Empty-Refill Exchange occurs when a tank is first emptied of coastal water and then refilled with oceanic water.

The National Invasive Species Act of 1996 (NISA) requests that vessels arriving from outside of the Exclusive Economic Zone (EEZ) voluntarily conduct open-ocean exchange—or use an approved alternate treatment of ballast water—of ballast tanks to be discharged in U.S. ports. More recently, individual states (e.g., California, Washington, and Maryland) have passed and implemented similar laws, sometimes making this management mandatory.

BWE is viewed generally as a temporary "stop-gap" measure to reduce the risk of invasions. It is a management strategy that many ships can implement immediately and does not require retrofitting or development of new technology. However, ballast exchange has

significant limitations. First, it is not always possible to safely conduct an exchange, which poses some risk to the structure and safety of vessels (especially under high seas). Second, even when performed, ballast exchange still leaves a residual of coastal organisms.

Efforts are now underway to develop and implement technological alternatives to BWE. Although many treatment possibilities are being explored (e.g., NRC 1996, Hallegraeff 1998, http:/www.invasions.si.edu), their evaluation is at a very early stage, and no alternative treatments have been approved.

At the present time, the U.S. Coast Guard (as directed by NISA) requires that alternative treatments be as effective as BWE. However, there exist no specific guidelines to assess the performance of treatments. Below, we present a conceptual framework for evaluation of alternative treatments and, using this framework, outline an approach (protocol) to measure the efficacy of a specific treatment system.

11.2.2 Conceptual Frame Work

The overall goal of ballast water treatment, including BWE, is to reduce the risk of invasion by limiting the delivery of viable propagules. Thus, assessing the efficacy of any treatment requires direct measures of its effect on the concentration and condition (or viability) of organisms transferred in ballast tanks.

There has been much discussion of desired treatment efficacy or "standard." At the present time, NISA requires treatment to be "as good as" BWE, making this the interim standard. However, for most vessels, the efficacy of BWE is uncertain, and we are just beginning to collect these data. In short, a specific numerical target has not yet been established.

Although the lack of specific standards may pose difficulties in advancing development of promising technologies, it does not create any technical impediments to measuring effects of ballast water treatments. Identification of specific standards is a high priority for many groups; especially those engaged in the development of technological treatments as well as state and federal management agencies. Standards provide the target or goal for treatment performance, which is assessed by quantitative measurements of effect, and should determine which measurements (and magnitude of effects) are required to demonstrate acceptable performance. However, the quantitative measurement of treatment effects can proceed whether standards exist or not.

Here, we wish to present a conceptual framework for testing the effects of ballast water treatment (specific aspects of study design for the ozonation project are given below in Sections 3-6). Our intent is to highlight key elements that should be addressed in the assessment of any treatment designed to reduce the risk of invasions associated with ballast tanks, drawing upon the extensive information that already exists about (a) ecology of marine and freshwater organisms, (b) design and operation of vessels, (c) BWE, and (d) experimental and statistical methods. Our primary focus is on testing of full-scale treatment systems aboard ships, addressing both the rationale and approach. Many of these same issues apply to "bench-top" tests, which may be performed as a pre-cursor to full-scale testing. Furthermore, the information presented here should assist in the development of standards.

Rationale: Identification of Key Variables

A first step in testing the effect of a ballast water treatment, or any other treatment, is to define as clearly as possible the key dependent and independent variables. Dependent or response variables include those attributes for which we wish to measure response to the treatment. Independent variables include both the treatment characteristics (e.g., magnitude and duration of treatment) as well as environmental conditions (or covariates) that may influence the response to treatment.

11.2.3 Response Variables

The appropriate dependent variable is some assay for reduction in capacity of organisms to invade successfully upon discharge from ballast tanks. Survivorship provides the most reliable measure in this regard. Treatments may also have sublethal effects, but these are more difficult to measure and interpret, since they may depend greatly upon environmental conditions. Although some measures of biological activity, such as concentrations of Adenosine Triphosphate (ATP) or chlorophyll *a*, can provide a quick general assay (National Research Council 1995), these do not permit analysis of variation in response likely to occur among the wide range of taxonomic groups present in ballast water (below).

Survivorship provides an unambiguous measure for the dependent variable that is strongly and explicitly associated with time. For example, it may take some time for mortality to occur, resulting in increased mortality over time due to some treatments. Alternatively, in the case of some microorganisms (e.g., bacteria), an initial decline in concentration due to treatment can be offset by an increase over time due to population growth of the residual organisms. As a result, assessment of a treatment should directly address possible temporal changes, especially where a "rebound" effect is possible, and may need to include measurements of initial effect of treatment (i.e., within hours to days of treatment conclusion) and final effect of treatment (i.e., at the end of a voyage of standard duration).

Which organisms should be used to test the effect of treatment? While this remains a topic of much discussion, the overall goal should be to include the full range of organism types and locations present in ballast tanks. To accomplish this goal, we suggest that tests should include representation across each of three different categories:

- **Habitats** – The ballast tank can be divided into at least 4 different habitat types, where organisms reside and treatment effects may vary, including: Planktonic, Epibenthic, Infaunal, and Sessile (including biofilms).
- **Taxonomic Groups** – Organisms from most major phyla have been reported in ballast tanks, and these may respond differently to treatment. Major taxonomic groups that have been reported in ballast tanks and should be included in treatment tests include: Viruses, Bacteria, Protists, Dinoflagellates, Diatoms, Crustaceans, Molluscs, Annelids, and Fishes.
- **Life History and Behavior** – Organisms often have many different life stages, and behaviors (such as geotaxis or phototaxis) can differ among species or life stages. Selection of taxa for tests should include multiple life stages and behaviors, especially those that are known to be most resistance to environmental changes (such as resting stages of dinoflagellates or zooplankton).

11.2.4 Treatment Characteristics

It is critical to measure the treatment characteristics at the time of testing, as the independent variable(s). Although most treatments are based upon some theoretical delivery of the treatment agent, actual delivery may differ, introducing error in the relationship between treatment and response. For example, ships that conduct BWE may run pumps for a specific amount of time, estimating a theoretical volume of water that is added in the process. However, we have learned that the actual amount can differ significantly, because pump rates depend upon head pressure, which changes as the tanks fill. Thus, the resulting data will greatly underestimate the efficacy of exchange, due to the disparity between theoretical versus actual volumes involved. Similarly, for any agent that is used to treat ballast water (e.g., biocides, ultraviolet radiation, pressure), it is necessary to measure the actual treatment levels delivered.

Where relevant, treatments should be characterized by measuring the treatment magnitude (e.g., dose, concentration, etc.) over time. For example, concentration of a biocide may (a) increase over time through addition and mixing with ballast water and (b) decrease over time after addition has ceased. The temporal dynamics of the biocide (concentration x exposure time) may greatly influence the outcome (survivorship). In a similar fashion, spatial variation in concentration may play an important role in outcome and should be explicitly addressed, as discussed below.

Also, where relevant, the creation and fate of chemical by-products that may be toxic should be measured over time for two purposes. First, some of these by-products may themselves influence survivorship, and tracking their fate may add interpretative power to the results. Second, because the treated ballast water is to be discharged, the potential risk of both the treatment agent and its by-products should be addressed.

11.2.5 Covariates

There are many reasons to expect the performance of ballast water treatments to vary with environmental and biological characteristics of ballast water, which will differ among source ports and seasons. For example, performance of a chemical agent or ultraviolet radiation may be influenced greatly by temperature, salinity, pH, turbidity, and biomass.

Key physical, chemical, and biological attributes of ballast water should, therefore, be measured for each test performed, and these measures should be repeated over time – since conditions may change in the course of a test. As a minimum, these attributes should include: Temperature, salinity, oxygen, pH, sediment load, and biomass.

Recognizing the potential importance of environmental and biological characteristics to treatment performance, a comprehensive testing program should attempt to include the full range of conditions encountered in the field (see “Phase III” below).

11.2.6 Approach: Methods and Analyses

Ballast water treatments will often undergo testing on multiple scales ranging from bench top and mesocosm laboratory tests to full-scale models that are installed aboard operational vessels. The smaller scale laboratory trials may provide a useful, cost-effective approach to explore the capability of a treatment, providing a “proof-of-concept” and further refinement for

increasingly larger scales. However, such laboratory experiments cannot replace full-scale field tests, where the potential for many variables (and interactions) may alter the expected outcome predicted by laboratory tests.

Each stage of testing involves the same basic issues. First, it is critical to define the treatment characteristics, the response variables (including both the specific organisms and effects assayed), and the environmental conditions. These issues, as discussed above, apply equally to any stage of testing. Second, the well-developed methods for experimental design and statistical analyses should be employed (see section on Data Analysis). Although the question of ballast water treatment effects is a specific one, the general principals and approaches for ecological research are relevant and necessary to draw robust conclusions.

In this section, we focus explicitly on the experimental design of testing ballast water treatment, focusing our attention on field-testing a full-scale model aboard a vessel. Although the same fundamental issues apply to laboratory testing, the field-testing presents perhaps the most challenging stage, due to the spatial scale complexity and scale of ballast tanks as well as the broad range of existing field conditions (biologically and environmentally).

11.2.7 Controls

A fundamental element of any experimental test includes the use of one or more control treatments, which controls for temporal and spatial changes that are independent of the experimental treatment. For example, it is clear that ballast water communities are dynamic, where many species exhibit large changes in abundance and condition over time (e.g., Smith et al. 1999, LaVoie 1999). In the absence of appropriate control treatment(s), changes in abundance or survivorship of a species may be erroneously attributed to the experimental treatment. In other words, the effect of a treatment must be assessed relative to changes in the control treatment.

In its simplest form, a controlled experiment to test the effect of ballast water treatment aboard a vessel should include (at least) one ballast water tank that is subjected to the experimental treatment and one tank that serves as a control. Any experimental tests should include as a minimum the following core elements:

- Ballast tanks for all treatments (including controls) should be filled simultaneously to obtain similar water samples and biota, because both environmental and biological characteristics can vary greatly over very short temporal and spatial scales;
- Dependent and independent variables should be measured for all ballast water tanks prior to treatment initiation, to control for initial differences that can exist among tanks (i.e., despite efforts to minimize these differences, substantial variation can still exist and must be accounted for in the analyses);
- All measures for the experimental treatment tank should also be conducted simultaneously in the control tank, using exactly the same methods.

Comparison to BWE

If one goal of the experiments is to compare the effect of a specific treatment to BWE, the experimental design should include one or more tanks dedicated to BWE treatment(s). There are multiple reasons to include such treatments:

1. BWE is the interim standard, as indicated in NISA;

2. The efficacy of BWE is unknown for most taxonomic groups and has only been measured on a few vessels to date;
3. The efficacy of BWE is likely to exhibit significant variation among vessels, taxonomic groups, and environmental conditions;
4. Thus, in the absence of a BWE treatment aboard the same vessel, comparisons with other treatments are not possible.

To include BWE treatment(s), it is preferable to perform these treatments at the same time as the other experimental and control treatments. As a minimum, one ballast water tank would be dedicated to BWE and filled simultaneous with the other tanks, to minimize the spatial and temporal variability in environmental / biological conditions. Further, the ballast exchange tank would be sampled initially and at some time point, parallel to the other treatments, allowing direct comparison(s) that control for temporal changes.

An alternate approach is to conduct independent exchange experiments. Although this can also be used to compare relative effects between exchange and another treatment, this approach suffers from many "uncontrolled" variables in the comparison and may require additional effort than the simultaneous experiments. Specifically, independent measures can be used to estimate the mean effect of treatments (exchange or alternate treatment) relative to controls. There are two shortcomings. First, the measures are collected on different species and under different conditions. To have confidence that observed differences are treatment effects, and not artifacts of different starting conditions, sufficient replication is necessary to span the same range of conditions and provide an adequate measure of variation for each data set; arguably, this would require many more replicate experimental tests to satisfy a critical audience. Second, conducting independent experiments requires a 33 % increase in treatments, regardless of the number of replicate experiments. Since both experiments (exchange or treatment) require the use of controls, there is an economy in conducting the exchange and alternate treatments together (n = 3 treatments, including one shared control treatment) compared to separately (n = 4 treatments, including one control for each treatment).

11.2.8 Replication

Although the identification of appropriate experimental treatments is often clear, issues of replication, independence, and generality can be confusing. We consider each experimental test to be the level of replication, providing data for one particular set of conditions (vessel, tank, source, and biota). For example, an experiment that includes 3 treatments – exchange, alternate treatment, and control ballast tanks – represents one replicate measure. Replicate samples taken within each tank (within or among times) cannot be considered independent measures and are used solely to measure variation and estimate mean conditions within a tank (see Sources of Variation, below). Comparisons can certainly be made among tanks, as planned, but these cannot be attributed to treatment effects without replicating the experiment. Replicate experiments provide a measure of variation observed in the relative treatment effects, allowing a test of whether consistent effects exist statistically.

Ideally, replicate experiments should be performed by alternating treatments among tanks, to control for effects due to tank differences (rather than treatment differences). If this

cannot be done, control measures are needed in each tank to test whether observed patterns may result from tank differences.

If strong treatment effects are observed, the generality of these results must be considered carefully. It is important to acknowledge the observed effects are specific to the particular vessel, tanks, and sources used for experimental tests. Although there may be good reasons to believe results are robust, we urge caution in such interpretation. We already know that environmental conditions and biota can vary widely among sources, and ballast tanks can vary tremendously in structure, size, shape, and operation. Such conditions may generate variation in effects among vessels and sources that must be tested to draw science-based conclusions about generality.

11.2.9 Sources of Variation

Performance of treatments will often be affected by many different variables. We have discussed some possible variation due to taxonomic group, habitat, life stages, and environmental conditions (above). Here, we wish to place this in the context of experimental design, considering sources of variation at various levels of the experimental design:

- Within-Tank
- Among Tanks
- Among Ships
- Among Experiments (Sources and Seasons)

To assess the effect of a treatment, it is necessary to address the considerable spatial variation that can exist in the distribution of organisms and effects. Although we have suggested use of habitats in measuring effects, there can still be a great amount of variation within each habitat. For example, some ballast tanks are divided into many different compartments. The initial concentration of organisms in the water column, and the effect of treatments, may differ among compartments. Even in ballast tanks that are large and relatively open, spatial variation can exist in both regards.

To address within-tank variation, all dependent and independent variables should be measured at multiple locations, horizontally and vertically distributed in each tank. The full range of habitat types (e.g., plankton, infauna, epibenthos, sessile) should be identified, and replicate samples should be collected in a stratified random manner within each habitat type. Such stratification should also include distance from treatment(s), if these are from point sources.

The number of replicate samples required per stratum will depend greatly upon the initial densities and the variation among samples. This approach is likely to be most effective for relatively small and abundant organisms. As abundance declines, or patchiness increases, it will become more difficult to test for treatment effects. For this reason, it may be useful to augment resident biota with additional "sentry" organisms, to represent the full spectrum of taxonomic groups and habitats (see "Methods" below).

Beyond within-tank variation, we should expect to see differences that occur among tanks, ships, and experimental conditions that must be addressed explicitly in experiments. To a large extent, we have already discussed each of these, and the implications for experimental

design are rather clear—separate measures (experiments) are needed to address each factor. Although this is rather overwhelming, when considering the full range of tank types X vessel types X source ports X seasons, there may be some practical approaches to expedite such analyses by focusing on extreme conditions as a conservative measure (see "Phases" below).

11.2.10 Methods

Using the general framework outlined above, we now wish to focus on sampling methods. There have been many different methods used in past studies of ballast water communities. The nexus of three features has driven these: logistical constraints (available access or time) for sampling; preferred sampling devices; and taxonomic focus. Access to ballast tanks varies tremendously among ships, and this can have an over-riding influence on sample type. Samples can be collected from hatches, cargo holds, sounding tubes, water mains, and empty tanks. Methods used to collect samples include plankton nets, Niskin bottles, pumps, settling tubes, video / camera, bottom cores, and culturable surfaces. The taxonomic focus has ranged from microorganisms to fishes. For the most part, past work tells us what has been possible and some of the constraints that have existed.

Ballast water treatment testing often requires a commitment of the vessel owner/operator that has not existed on many prior ballast water studies. Specifically, treatments are being installed aboard vessels with the explicit purpose of conducting field tests, creating an opportunity for improved sampling access. In past studies, access was granted on vessels, often boarded with little forewarning or knowledge, and samples were collected where possible. Thus, as part of the development phase of any project, sufficient attention should be given to both experimental design and sampling strategy, to maximize the information needed (by the owner/operator) for adequate evaluation.

At the design phase, we recommend creation of a "sampling matrix" that identifies the sampling locations, sample types, and sample frequencies. This, in turn, can help drive the overall design and increase the quality of needed data.

In general, we suggest the following requirements:

- Access to all habitat types and strata (as discussed above) is critical;
- Access should exist for replicate locations within each sampling stratum;
- Access should include hatches for use of nets and "sentry" organisms;
- Facilities are needed for shipboard processing and analyses of samples.

As discussed above, the introduction of "sentry" organisms into ballast tanks may greatly enhance the quality of data on multiple fronts. First, inoculation can be used to increase the abundance of free-ranging organisms (from bacteria and viruses to zooplankton). Second, use of standard inocula across ships provides for standard measures. We should expect a wide variation in the resident organisms that are entrained, whereas the inoculation of organisms resolves this problem, allowing each experiment (among within or among ships) to include the same measures. Third, inoculation of caged organisms may also provide standard measures for organism types or habitats that are extremely difficult to measure, patchy in nature, or mobile.

Thus, we suggest ballast treatment experiments aboard vessels take advantage (in the design phase) of three different sources of biota:

1. Resident biota that is entrained during normal ballasting operations;
2. Inoculated biota that is free-ranging in the ballast tanks;
3. Caged sentries that are used to measure effects on particular problematic taxa or habitats.

An example of using mixed sources of biota for a design strategy is outlined here:

- Resident biota may be most effective at measuring effects for small organisms such as bacteria, viruses, protists, diatoms, and dinoflagellates. These could be collected by whole water samples (pumps or Niskin bottles). In addition, resident biota could be used for some zooplankton, especially copepods. These are best collected by net tow through the water column, both concentrating the organisms and integrating small-scale spatial variation, which is likely to be greater for these organisms than the smaller ones sampled by whole water.
- Many zooplankters will not be effectively sampled with either method—due to low densities—such as molluscs, mysids, worms, and crabs. These could be introduced either as free-ranging organisms or as caged organisms. The latter approach would be most effective for fishes or epibenthic organisms, and cages should be deployed at multiple locations to assess the effect of a treatment of survivorship.
- Similar sentries could be used for infaunal communities (e.g., buckets of sediment inoculated with worms and bivalves) and sessile invertebrates (e.g., biofilms or invertebrates).

11.2.11 Phases

There are clearly a large number of parameters and variables to consider in evaluation of ballast water treatments, and we have discussed many of the associated complexities. While this can be rather overwhelming, it is helpful to consider a graded approach to testing that consists of multiple phases of field-testing.

The first phase is to perform an initial series of controlled experiments controlling for many parameters and utilizing a limited suite of measures. For example, 4 - 6 replicate experiments may include measures of bacteria and sentry organisms at a few locations, combined with measurements of the independent variables. The purpose of this phase is to determine whether potential effects are detectable. This is a "proof of concept" phase. If no effects are demonstrable, with a coarse level analysis, it may not be worth the expenditure to proceed. If effects are observed, further testing is needed to measure effects across a broader range of taxa, habitats, life stages, and conditions. Sections 5 and 6 below outline our approach to this Phase I study.

The second phase is to expand the scope of measures to additional taxonomic groups and habitats, include much greater level of spatial (within-tank) replication.

The third phase it to expand the scope to include the full suite of measures across multiple source ports, seasons, and operations. Although this phase is daunting, when considering the number of permutations, one approach would be to select extremes (e.g., high sediment load and high biomass), where the performance of the treatment is likely to be least effective. This provides quickly the full range of possible responses to inform development of additional testing strategies.

Statistical Analyses

There are many statistical approaches that can be used to analyze the resulting data. Our preference is to use ANOVA, examining the change in survivorship in experimental versus control and exchange treatments. Since the treatments are paired in each experiment, a paired test is most appropriate, using experiment as the level of replication.

Additional analyses may include multivariate analyses to test for association between dependent and independent variables.

Throughout, it is key to include a rigorous statistical approach, clearly identifying not only mean effects but also the level of variation observed within tank and experiments. This has not always been presented for existing tests, creating confusion about the validity of interpretation presented.